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B. The Amendments:

The foregoing amendments to the claims are presented in response to the Examiner's comments in the Office Action and to more clearly and distinctly claim what Applicants believe the invention to be. Applicants do not believe that the foregoing amendments add any new matter to the specification and respectfully request that these amendments be added prior to the consideration of the following remarks. As the total number of claims remains the same as that already paid for and no additional independent claims are added by the foregoing amendments, Applicants do not believe that any fee is associated with the foregoing amendment.

C. Rejection Pursuant to 35 U.S.C. 112 Second Paragraph:

Claims 13-18 and 34-39 are rejected pursuant to 35 U.S.C. 112 second paragraph as indefinite.

Claims 13-18 stand rejected indefinite an confusing in relating to the increased level of interferon alpha without precedent relating to the pre-existing level. Applicants have amended the scope of the claim 13 to recite that the interferon alpha polypeptide is expressed at a therapeutically effective amount and no longer relates to the increased level but merely to the expression of the protein in the cell. Applicants believe that this obviates this ground of rejection.

Applicants note the Examiner's rejection of Claim 18 as redundant and have cancelled this claim obviating this ground of rejection.

Applicants have amended claim 34 to recite that a therapeutic effect is achieved thus obviating this ground of rejection.

D. Rejection Pursuant to 35 U.S.C. 112 First Paragraph:

There are three requirements to comply with the provisions of 35 U.S.C. 112, first paragraph: (1) the written description requirement; (2) the enablement requirement; and (3) the best mode requirement. In this instance, the *Examiner* has indicated that the pending claims fail to comply with the written description requirement stating:

> Claims 1-39 are rejected under 35 U.S.C. 112, first paragraph as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention."

The *Examiner* has set forth numerous positions on this issue, the underlying theme of which is that as of the time of filing, the state of the art of gene therapy was so poorly understood that the specification must provide exquisite detail regarding every facet of the potential clinical application in order to provide those of skill in the art with sufficient guidance to practice the claimed methods. At page 3 of the Office Action, the *Examiner* states:

> the specification does not provide any guidance as to the level of gene expression required, the number of transduced cells needed, the route and time course of administration, the site of administration, when, where, or for long the IFN-alpha gene should be expressed, the frequency of administration of the IFN-alpha encoding gene therapy vector required, or in some embodiments, the intended target tissue, for treatment of any pathological condition in an immunocompetent model

Secondly, because the compositions described are allegedly useful solely for gene therapy and because such use was not enabled, the compositions (vectors and pharmaceutical formulations) are similarly not enabled. The Examiner requires of Applicants a level of specificity in the specification that is contrary to the statutory requirements and in conflict with the standard that is routinely applied by the United States Patent and Trademark Office in other gene therapy applications. Applicants believe that the teaching of the specification provides those of skill in the art with the requisite guidance to make and use the compositions of the present invention in accordance with the Wands factors.

Applicants note that the *Examiner* has not made a rejection of the pending claims pursuant to 35 U.S.C. 101. The provisions of 35 U.S.C. 112, first paragraph address different concerns than those addressed by 35 U.S.C. 101. As stated in MPEP2107 ("General Principles Regarding Utility Rejections"):

> It is important to recognize that 35 U.S.C. 112, first paragraph, addresses matters other than those related to the question of whether or not an invention lacks utility. These matters include whether the claims are fully supported by the disclosure (In re Vaeck, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991)), whether the applicant has provided an enabling disclosure of the claimed subject matter (In re Wright, 999 F.2d 1557, 1561-1562, 27 USPQ2d 1510, 1513

> (Fed. Cir. 1993)), whether the applicant has provided an adequate written description of the invention and whether the applicant has disclosed the best mode of practicing the claimed invention (Chemcast Corp. v. Arco Indus. Corp., 913 F.2d 923, 927-928, 16 USPQ2d 1033, 1036-1037 (Fed. Cir. 1990). See also Transco Products Inc. v. Performance Contracting Inc., 38 F.3d 551, 32 USPQ2d 1077 (Fed. Cir. 1994); Glaxo Inc. v. Novopharm Ltd. 52 F.3d 1043, 34 USPQ2d 1565 (Fed. Cir. 1995)). The fact that an applicant has disclosed a specific utility for an invention and provided a credible basis supporting that specific utility does not provide a basis for concluding that the claims comply with all the requirements of 35 U.S.C. 112, first paragraph. For example, if an applicant has claimed a process of treating a certain disease condition with a certain compound and provided a credible basis for asserting that the compound is useful in that regard, but to actually practice the invention as claimed a person skilled in the relevant art would have to engage in an undue amount of experimentation, the claim may be defective under 35 U.S.C. 112, but not 35 U.S.C. 101. To avoid confusion during examination, any rejection under 35 U.S.C. 112, first paragraph, based on grounds other than "lack of utility" should be imposed separately from any rejection imposed due to "lack of utility" under 35 U.S.C. 101 and 35 U.S.C. 112, first paragraph.

Consequently, in accordance with the provisions of MPEP 707.07(g) regarding the impropriety of piecemeal examination, Applicants believe that the *Examiner* is not questioning the utility under 35 U.S.C. 101 of the claimed compositions but merely whether the specification provides sufficient guidance to those of skill in the art to make and use the compositions in accordance with the claimed methods.

The test of enablement is whether one of ordinary skill in the art would be able to practice the claimed invention without undue experimentation. *Mineral Separation v. Hyde*, 242 U.S. 261, 270 (1916). Although there are many factors to be considered in determining whether or not the level of experimentation required of those of skill in the art would be undue, *In re Wands* set forth those factors which have been routinely employed to determine whether the level of experimentation is undue:

1. The breadth of the claims;
2. The nature of the invention;
3. The state of the prior art;
4. The level of one of ordinary skill;
5. The level of predictability in the art;
6. The amount of direction provided by the inventor;

7. The existence of working examples; and
8. The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) In evaluating the factors, the MPEP provides that:

> It is improper to conclude that a disclosure is not enabling based on an analysis of only one of the above factors while ignoring one or more of the others. The examiner's analysis must consider all the evidence related to each of these factors, and any conclusion of non-enablement must be based on the evidence as a whole. 858 F.2d at 737, 740, 8 USPQ2d at 1404, 1407.

Conseqeuntly, applicants will address the *Examiner*'s positions in regard to 35 U.S.C. 112, first paragraph within the framework of the *Wands* factors.

1. The breadth of the claims:

The Examiner indicates that the claims are broad encompassing all therapeutic utilities. Although the applicants agree that the present compositions of the present invention are useful in a variety of contexts, it is clear from the specification that the compositions are being employed to treat diseases which are amenable to treatment by IFN-alpha protein. See specification at pages 19 and 20. The claims have been amended to recite this limitation. Although the claim does encompass a number of potential clinical applications, the utility of IFN-alpha in such clincial applications has been established in the scientific literature. Therefore, applicants do not believe that the claim is particularly broad because it is limited to the treatment of those disease states already shown to be amenable to treatment with the interferon-alpha protein alone.

2. The nature of the invention;

The nature of the invention relates to methods of treatment of human beings through the use of recombinant nucleic acid sequences to deliver and express interferon alpha polypeptides to facilitate the treatment of a disease state amenable to treatment with interferon alpha polypeptides.

3. The state of the prior art:

The prior art relating to the practice of the present invention relates not only to gene therapy but also to the state of the prior art surrounding the clinical applications of alpha-interferons. Although gene therapy is a relatively new field, there are a significant number of issued United States patents relating to this technology in advance of the filing date of the present application. A sampling of these patents and the scope of their corresponding claims is discussed below. Additionally, the state of the art regarding interferons is well established. Interferons have been in routine clinical use for more than 15 years. Various forms of recombinant interferon-alpha polypeptides are commercially available such as Intron® (interferon alpha 2b recombinant, Schering-Plough Corporation), PEG-Intron® (a sustained acting PEGylated form of Intron®), and Infergen® (interferon alfacon-1, Amgen Corporation). The specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public. Consequently, based on the substantial quantity of prior art regarding the delivery of nucleic acids available to the skilled artisan combined with the enormous body of scientific literature and clinical experience regarding interferon alpha proteins, the level of prior art in this field on which the skilled artisan might rely is substantially greater than is represented by the Examiner.

4. The level of one of ordinary skill;

The level of explicit guidance required in the specification in order to comply with the enablement requirement is inversely proportional to the level of those skilled in the art to which the invention pertains. The field of molecular biology is generally considered to be one of the most highly skilled fields. A number of decisions have held that the ordinarily skilled artisan in the field of biotechnology would generally be a person with a doctorate degree and likely one or more post-doctoral fellowships. The clinical application of recombinant gene therapy is a particularly specialized group generally consisting of individuals practicing medicine at large university research hospitals. The individuals who would generally be employing the vectors of the present invention are particularly cognizant of the scientific literature in the field. The number of "physician sponsored INDs" for clinical trials of recombinant viral vectors approved

by the FDA and receiving NIH funding attests to the fact that clinicians in this field are of a particularly high caliber.

Consequently, the ordinarily skilled artisan in this field would be quite sophisticated. Given the very high level of training in this field, the ready access to clinical expertise, the volume of scientific literature available it would not require undue experimentation for the ordinarily skilled artisan to employ the compositions of the present invention in the clinical setting. The fact that there are over 200 ongoing clinical trials involving gene transfer gene therapy protocols demonstrates that the level of skill in the art is sufficient to enable the ordinarily skilled artisan in this field to practice the technology without undue difficulty.

The level of predictability in the field of gene therapy is difficult to ascertain because due to the newness of the technology most of the products are still in clinical trials. The amount of guidance or direction needed to enable the invention is inversely related to the amount of knowledge in the state of the art as well as the predictability of the art. MPEP 2164.03 *citing In re Fisher* 166 USPQ 18, 24 (CCPA 1970). Whether or not any agent will be effective in the treatment of human beings bears a certain degree of uncertainty. However, the fact that most compounds which are proposed for clinical investigation do not result in FDA marketing approval has not been a bar to patentability. Consequently, in the case of gene therapy agents, the focus on the degree of teaching required to enable the scope of the claims more properly focuses on the level of knowledge in the art. Applicant has discussed elsewhere in this Response the extraordinarily high level of skill in this art. Applicants believe that the high levels of skill and knowledge in this art are sufficiently such that a rather limited disclosure would enable one of skill in the art to practice the full scope the claimed invention.

The applicants also wish to observe that the quantity of experimentation considered "undue experimentation" is a moving target. The new technologies that have been introduced to the biotechnology research community have dramatically altered the amount of time involved in performing certain experiments. For example, in the 1980's, cloning a gene was a laborious process requiring weeks (if not months) of work. With the advent of PCR technology, one is generally able to clone a gene from a commercially available library in a matter of days. The extensive processes to create "humanized" or "CDR grafted" antibodies having reduced immunological reactivity has been eliminated by the ability to obtain fully human antibodies

from transgenic mice. One is now able to obtain fully human antibodies using technology commercially available from companies such as Abgenix, Inc. (Fremont CA) or Cambridge Antibody Therapeutics (Cambridge MA). The advent of genomics and proteomics technologies coupled with sophisticated assay systems enables the ordinarily skilled artisan to generate a tremendous amount of data regarding the potential activity of a compound in a very short period of time. The "chip" or "microarray" technology to evaluate the response of genes in multiple cell types in response to a stimulus did not exist 10 years ago. However, a scientist may now rapidly evaluate the response of many thousands of genes in a cell in response to a potential pharmaceutical. This technology is available to the scientific community from companies such as Incyte Therapeutics (Palo Alto, CA) and Millenium Pharmaceuticals (Boston MA).

As a result, the skilled artisan is able to generate a significant quantity of data with relative ease. Using these new technologies, agents that are proposed for clinical investigation at the present time have significantly more data to support their utility than at any time in the past. Compounds that were brought to the clinic in the 1970's had comparatively little background information regarding mechanism of action. Out of this background grew a considerable body of case law indicating that therapeutic activity was unpredictable. The Examiner must take into consideration the time context of certain case law indicating that certain procedures involved "undue experimentation" or that a technology was deemed "unpredictable." The mere fact that gene therapy is a relatively new technology in terms of years does not mean that it is not supported by a wealth of scientific data.

5. The Level of Predictability In the Art:

The Examiner indicates that by virtue of being related to the field of gene therapy, that the invention is unpredictable per se requiring particularly detailed description. In support of the "unpredictability" the Examiner cites Crystal (1995), Miller and Vile (1995), Deonorian (1998) and Verma, et al. (1997). The Applicants would note that all of these references are either review articles or commentaries and do not fairly reflect the state of the art at the time the present invention was made. The pace of development of the field of gene therapy has been very rapid and three of these references reflect work much earlier than the date of the application and do not even provide a fair characterization of the state of the art as can be seen from other publications,

some of which are by the same authors. First, the Miller and Vile reference is a review published in 1995 of prior literature. The most recent reference reviewed in this paper is from 1994 -- of which only 4 of the 71 cited references is even from 1994. Similarly, Dr. Ronald Crystal's Science article is another 1995 review of the published literature. It is particularly interesting to note Dr. Crystal's comments in view of the following issued United States patents of which Dr. Crystal is an inventor all of which relate to gene therapy:

Patent No.	Title
6,165,754	Method of expressing an exogenous nucleic acid
6,159,950	Method of modulating hair growth
6,153,435	Nucleic acid that encodes a chimeric adenoviral coat protein
6,127,525	Chimeric adenoviral coat protein and methods of using same
6,013,638	Adenovirus comprising deletions on the E1A, E1B and E3 regions for transfer of genes to the lung
5,997,509	Minimally invasive gene therapy delivery device and method
5,928,944	Method of adenoviral-medicated cell transfection
5,869,037	Adenoviral-mediated gene transfer to adipocytes
5,837,511	Non-group C adenoviral vectors

It is clear that the opinions expressed by Dr. Crystal in the cited reference are contradicted by Dr. Crystal's opinions expressed in the nine issued United States Patents relating to gene therapy of which he is co-inventor. Similarly, Dr. Verma's opinion must have changed over time since writing the cited reference as Dr. Verma is listed as a co-inventor of three issued United States Patents relating to gene therapy;

Patent No.	Title
6,013,516	Vector and method of use for nucleic acid delivery to non-dividing cells
6,008,323	Transdominant negative proto-oncogene
5,470,736	Transdominant negative proto-oncogene

In view of the Examiner's position citing Dr. Verma's position on targeting please note the scope of claims 8 through 14 of the '736 patent which relate to targeted delivery of the nucleic acids through the use of various targeting ligands. Also note that the '736 patent issued based on a continuation of the priority application filed on June 10, 1991.

Finally, the Deonarian reference does state, as the Examiner quotes, that "one of the main obstacles to fulfilling the promise [of gene therapy] is in the ability to target a gene to a significant population of cells and expressing it at adequate levels for a long enough period of time." However, the reference as a whole is directly to the contrary focusing on the promise of non-viral polyplex targeted delivery of DNA. Further in the same paragraph, the author states:

> These complex are able to deliver genes to cells in a receptor-specific manner, without any viral DNA sequences of packaging constraints, There are now many ligand/receptor systems under investigation, each one demonstrating successful gene transfer with a higher level of tissue specificity than viruses can offer. This review describes most of these systems and looks ahead to an era where cell specific gene delivery man be a main stream gene therapy, treatment modality.

And at page 65,

> Research into tissue-specific targeting of tissues such as the liver, bone marrow stem cells and macrophages is well advanced, with good prospects for clinical testing. Tumour targeting of genes is also progressing well, basically following the same lines of receptor targets as previous immunotoxin research. Genetic delivery of toxins to tumors may prove to be more effective than immunotoxins.

Consequently, it is clear that Deoranian reference, taken as a whole, endorses rather than questions the success of gene therapy approaches.

Therefore, applicants believe that they have rebutted the Examiner's allegation that gene therapy is "unpredictable" in reliance these four references in support of her position. While applicants will acknowlege that whether or not any agent will be effective in the treatment of human beings bears a certain degree of uncertainty. However, the fact that most compounds which are proposed for clinical investigation do not result in FDA marketing approval has not been a bar to patentability. Consequently, in the case of gene therapy agents, the focus on the degree of teaching required to enable the scope of the claims more properly focuses on the level of knowledge in the art. Applicants believe that the high levels of skill and knowledge in this art are sufficiently such that a rather limited disclosure would enable one of skill in the art to practice the full scope the claimed invention.

6. The amount of direction provided by the inventor;

The present specification does provide a significant level of teaching at least equal to and in most cases substantially in excess of other disclosures supporting the issuance of gene therapy claims. The present application does provide detailed descriptions of the types of Diseases amenable to treatment at page 19, line 30 through page 20, line 20. Specific information is provided regarding dosage regimens at page 20, line 12 through page 22 line 25.

7. The existence of working examples:

In the present invention the specification does provide working examples demonstrating the therapeutic utility of the claimed invention. In particular, the specification provides data in a mouse model of cancer. The *Examiner* has rejected these examples as consituting "working examples" because they do not correlate with the scope of the claimed invention. As provided in the MPEP 2164.02,

> An in vitro or in vivo animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. If there is no correlation, then the examples do not constitute "working examples.

In this regard the *Examiner* focuses on the fact that the model used was performed in an immunodeficient BALb-c mouse and that therefore does not accurately predict the effect of the claimed method in an immunocompetent host. This position is echoed throughout the Office Action at pages 3,4,6 and 7. The *Examiner* questions whether or not it is possible to administer a recombinant adenovirus to a subject (singularly or repeatedly) without inducing a neutralizing immune response. The Examiner's position in this regard is in error.

There are numerous examples of the repeated administration of recombinant adenoviral vectors to immunocompetent human subjects demonstrating that it is possible to administer the compositions of the present invention to an immunocompetent host without neutralization by the innate or induced immune response. For example, it is well documented in the scientific literature that replication deficient adenoviruses encoding the p53 tumor suppressor gene have been administered to human beings in ovarian cancer clinical trials in dosages of greater than 10^{13} viral particles per dose. This dose has been administered and is well tolerated throughout a three

week dosage regimen of 5 daily doses resulting in the administration of more than 10^{14} viral particles over a period of three weeks. This work is presented in Nielsen, *et al.* (Cancer Gene Therapy (1998) 5:52-63) a copy of which is attached hereto as Exhibit A. As can be seen from the data presented, a variety of gene therapy clinical trials have used multiple dosing strategies and have resulted in prolonged transgene expression. Additionally, Nielsen, et al (Adenoviruses: Basic Biology to Gene Therapy (1999) R.G.Landes, Co., P.Seth, ed., Chapter 32), a copy of which is also attached as Exhibit B, particularly state that the recombinant adenovirus was administered to human subjects over multiple doses, that a immunological response was observed, but that "an acceptable safety profile and transgene expression were both confirmed."

Additionally, wild-type and attenuated viruses have been administered to human beings in additional clinical trials and clinical experiments. Early reports in the literature demonstrate the use of wild-type vectors for therapeutic use. Although these studies are not well characterized by modern standards, they do demonstrate the ability to use replication competent, indeed fully wild-type, vectors *in vivo*. More recent clinical experiments conducted by Onyx Pharmaceuticals, Inc. with the ONYX-015 virus (a recombinant adenovirus containing a deletion in the E1b55K region) are currently under clinical investigation in human clinical trials for the treatment of head and neck cancer. The clinical protocols and results of these studies have been published in the scientific literature and presented at numerous scientific conferences. These scientific studies in human beings provide a great deal of guidance to the skilled artisan in the specifics of route of administration and dosage regimens which are acceptable in the human being. In particular, Nemuniatis, et al. (J. Clin. Oncology (2001) 19:289-298) a copy of which is attached to this Response as Exhibit C provides clinical experience regarding ONYX-015 and the relationship to neutralizing antibodies. As stated at page 294-295, although the majority of patients demonstrated the presence of neutralizing antibodies following treatment, "[t]here was no correclation of baseline titer levels to tumor response, time to local progression progression-free survival duration or overall survival." In column 2 of page 295, Dr. Nemunaitis discusses the immunity question squarely indicating that the pre-existing immune response is not a bar to the use of recombinant adenoviral vectors in human beings. Another paper by Nemunaitis, et al (Cancer Research (2001) 606359-66) a copy of which is attached as Exhibit D to this Response,

clearly states "[h]igh neutralizing antibody titers did not prevent infection and/or replication within tumors."

The Examiner's position that the use of the compositions of the present invention in an immuno-competent host, especially an immuno-competent human host, would result in clearance and inactivation of the gene therapy agent is directly contradicted by the human clinical evidence. Therefore, the examples of *in vivo* efficacy supplied in the instant application do provide correlation to the clinical utility of the compositions of the present invention.

8. The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

As previously discussed, the present application does provide a significant quantity of information regarding use of the compositions of the present invention in the context of the practice of the methods of the claimed. Applicants believe that the level of disclosure provided herein in combination with the high level of skill in the art would enable the skilled artisan in the field to practice the claimed methods and effectively use the claimed compositions. However, the *Examiner* has taken the position that the content of the disclosure should be exceptionally detailed in this field because of the alleged unpredictability of the art. Applicants believe that the Examiner is applying a standard which is improper and not reflective of the Office's position regarding the level of teaching required to enable a gene therapy claim.

For example, Applicants would bring to the Examiner's attention Cuthbertson, United States Patent No. 6,204,251 issued March 20, 2001 based on a priority specification filed October 31, 1994. Claim 1 of this issued patent reads:

> 1. A method of treating ocular disease comprising incorporating exogenous nucleic acid into an in situ ocular cell under conditions permissive for the uptake of said exogenous nucleic acid, said exogenous nucleic acid encoding a protein associated with said ocular disease, whereby said exogenous nucleic acid is expressed and said disease is treated.

This claim is not restricted to a particular disease state, protein to be expressed, method of administration or type of vector. In the Office Action the Examiner takes the position that the present specification is deficient in that it fails to provide any guidance as to the level of gene expression required, the number of transduced cells needed, the route and time course of

administration, the site of administration, etc. When comparing the claims of Cuthbertson in view of the specification, it is clear that the Office required very little guidance relating to dosing, frequency of administration, etc. and certainly not commensurate with the scope of the disclosure being required of the present applicants.

The Office's position is reflected in the scope of the claims other issued United States Patents relating to gene therapy are not limited in scope regarding particular routes of administration or to narrow disease states. The following is a review of the claims of issued United States patents and the corresponding specifications to illustrate this point. Hammond, et al. United States Patent No. 6,174,871 entitled "Gene therapies for enhancing cardiac function" issued January 16, 2001 based on a specification filed June 7, 1995 provides as claim 1:

> 1. A method for treating a heart disease, wherein said heart disease includes a symptom of myocardial ischemia, by increasing blood flow to the myocardium of a patient, comprising delivering a replication-deficient adenovirus vector to the myocardium by intracoronary injection directly into the lumen of one or more coronary arteries, said vector comprising a gene the expression of which causes production of an angiogenic protein or peptide, thereby increasing blood flow to the myocardium.

and claim 20 of the same patent provides:

> 20. A method for treating a heart disease, wherein said heart disease includes a symptom of myocardial ischemia, by increasing the contractile function of the myocardium of a patient, comprising delivering a replication-deficient adenovirus vector to the myocardium by intracoronary injection directly into the lumen of one or more coronary arteries, said vector comprising a gene the expression of which causes production of an angiogenic protein or peptide, thereby increasing the contractile function of the myocardium.

A brief review of the specification will demonstrate that this patent contains relatively little guidance relating to the particular treatment protocols and data from a rat and porcine model of myocardial ischemia, but nevertheless was found sufficient to support a claim unrestricted by any particular dosage regimen or any particular angiogenic protein or peptide.

A second patent has also issued based on this specification (Hammond , et al. United States Patent No. 6,100,242 issued August 8, 2000 entitled "Gene therapies for enhancing cardiac function") which provides as claim 1:

> 1. A method for increasing contractile function in the heart of a patient, comprising delivering a transgene encoding an angiogenic protein or peptide to the myocardium of

> the patient by introducing a replication-deficient adenovirus vector comprising the transgene into the lumen of a coronary artery supplying blood to the myocardium, whereby the transgene is delivered to the myocardium and expressed and contractile function in the heart is increased.

This is particular interesting as this claim, unlike the claim of the '871 patent is not limited by the method of administration (e.g. intracoronary injection) nor to any identifiable disease state but merely results in the increase in contractile function of the heart of a patient (presumably human). The level of protein to be expressed is absent from this description, the quantity or type of vector is similarly absent from this claim, as is the identity of angiogenic peptide or protein.

Similarly, Henderson, et al, United States Patent No. 5,871,726 entitled "Tissue specific and tumor growth suppression by adenovirus comprising prostate specific antigen" issued February 16, 1999 based on a specification filed June 26, 1996 provides claims to a vector in claim 1:

> 1. An adenovirus vector comprising an adenovirus gene essential for propagation under transcriptional control of a prostate specific response element, said prostate cell specific response element comprising an enhancer specific for prostate specific antigen and a promoter.

and an *in vivo* method of use of such vector in claim 30:

> 30. A method for suppressing tumor growth comprising introducing the adenovirus vector of claim 1 into a tumor cell expressing prostate specific antigen (PSA), wherein introduction of the adenovirus vector results in suppression of tumor growth.

The sum total of the guidance regarding the clinical application of this vector is contained in two paragraphs:

> The modified viruses may be delivered to the target cell in a variety of ways, depending upon whether the cells are in culture, ex vivo or in vivo. For the prostate, for the most part, the cells will be delivered in vivo. Delivery can be achieved in a variety of ways, employing liposomes, direct injection, catheters, intravenpis inhalation, topical applications, etc. Due to the high efficiency of transfection of adenoviruses, one can achieve a high level of modified cells. In the case of neoplasia, where toxins are produced, the toxins will be released locally, so as to affect cells which may not have been successfully transfected. In this manner, one can specifically eliminate the neoplastic cells, without significant effect on the normal cells. In addition, expression of adenovirus proteins will serve to activate the immune system against the target cells. Finally, proliferation of the adenovirus in a host cell will lead to cell death.

> The adenovirus may be administered in an appropriate physiologically acceptable carrier at a dose of about 10^4 to 10^{11}. The multiplicity of infection will generally be in the range of about 0.001 to 100. The viruses may be administered one or more times, depending upon the immune response potential of the host. If necessary, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune response.

This is considerably less guidance than that provided by the instant specification, yet the claim of Henderson, et al. is not restricted to any delivery system, dosage range, etc. as suggested by the Examiner.

Morsy, et al., United States Patent No. 6,001,816 entitled "Gene therapy for leptin deficiency" issued December 14, 1999 based on a provisional specification filed June 20 1996 provides as claim 1:

> 1. A method of treating obesity in a mammal having a deficiency in functional leptin comprising administering intravenously to the mammal an adenoviral vector comprising a DNA sequence encoding a leptin operably linked to a promoter and expressing the DNA sequence, wherein the mammal exhibits a decrease in body weight, a decrease in serum glucose levels and/or a decrease in serum insulin levels.

The sum total of the Morsy, et al. specification's teaching regarding the practice of the method of the invention is contained in three paragraphs:

> If a vector is chosen as the delivery vehicle for the obesity regulating gene, it may be any vector which allows expression of the gene in the host cells. It is preferable if the vector also is one which is capable of integrating into the host genome, so that the gene can be expressed permanently, but this is not required. In cases where the vector does not integrate into the host genome, the expression of the gene may be transient rather than permanent.
>
> One vector which is suitable for transient expression of the ob gene is an adenovirus which has a deletion in the El gene. Such vectors are known, as taught in the aforementioned WO 95/00655 and Mitani et al., 1995 publications. These viruses preferentially infect hepatocytes, where they persist for approximately 3-4 weeks after the initial infection. While in the hepatocytes, these viruses can express the heterologous gene.
>
> The vector is administered to the host, generally by IV injection. Suitable titers will depend on a number of factors, such as the particular vector chosen, the host, strength of promoter used and the severity of the disease being treated. For mice, an adenovirus vector is preferably administered as an injection at a dose range of

> from about 5.0×10^6 to about 10×10^6 plaque forming units (PFU) per gram body weight. Preferred dosages range from at least about $6\text{-}9 \times 10^6$ PFU/gm body weight, and more preferred is from at least about $6.7\text{-}8.6 \times 10^6$ PFU/gm body weight (equivalent to approximately at least $1\text{-}5 \times 10^8$ PFU for mice).

Additionally, the disclosure was supported by a single mouse experiment. However, the claims are not limited to any particular vector, any particular method of administration, any specific promoter, any particular level of expression or even any particular mammal as the Examiner suggests is necessary in the instant case.

Another example is Woo, et al. United States Patent No. 6066624 issued May 23, 2000 entitled "Gene therapy for solid tumors using adenoviral vectors comprising suicide genes and cytokine genes" based on a specification filed February 15, 1993 (claiming priority to an application filed in 1993) which provides as claim 1:

1. A method of causing regression of a solid tumor in a mammal, comprising the steps of:
- administering an adenoviral vector directly into said tumor, wherein said vector is comprised of a DNA sequence encoding a suicide gene, and one or more cytokine genes, wherein said genes are operably linked to a promoter, and wherein said tumor expresses said suicide gene and said one or more cytokine genes; and
- administering a prodrug in amounts sufficient to cause regression of said tumor when said prodrug is converted to a toxic compound by said suicide gene.

The teaching of the Woo, et al. specification regarding the practice of the invention is contained in two paragraphs:

> Ganciclovir may be administered readily by a person having ordinary skill in this art. A person with ordinary skill would readily be able to determine the most appropriate dose and route for the administration of ganciclovir. Preferably, ganciclovir is administered in a dose of from about 1-20 mg/day/kg body weight. Preferably, acyclovir is administered in a dose of from about 1-100 mg/day/kg body weight and FIAU is administered in a dose of from about 1-50 mg/day/kg body weight.
>
> In another method of the present invention, an adenovirus containing a cytokine gene sequence can be driven by various promoters including Rous Sarcoma Virus-Long Terminal Repeat, cytomegalovirus promoter, murine leukemia virus LTR, simian virus 40 early and late promoters, and herpes simplex virus thymidine kinase. This cytokine gene-containing adenovirus can then be co-administered with the suicide gene-containing adenovirus, to effect a "combination gene therapy."

Applicants can provide additional example, but believe the foregoing adequately illustrates the point that the standard applied by the USPTO is substantially different that the standard attempting to be applied by the Examiner in the present application. Applicants therefore submit that the standard being applied by the Examiner in this instance is unduly restrictive and improper and that the teaching of the specification is commensurate with the scope of the claims in accordance with the standard applied in such cases.

E. Rejection of Claims 1-4, 13, 14, 17 and 18 Pursuant to 35 U.S.C. 102(e):

Claims 1-4, 13, 14, 17 and 18 (claims 10 and 11 were cancelled by amendment) stand rejected in view of Chiou, et al (United States Patent No. 6,069,133). The standard for anticipation pursuant to 35 U.S.C. 102(e) is the same as that for 35 U.S.C. 102(b), i.e. that the specifically claimed invention be described in the single prior art reference. Applicants would draw the attention of the Examiner to the foregoing amendments to the claims entered in this Response. In claims 1 (from which claims 2- 4 and 6-9 depend) and claim 13 (from which claims 14-17 depend) applicants have amended the scope of the claim to recite that the interferon polypeptide is expressed from a viral vector. The specification and claims of Chiou, et al. clearly relate to cationic polyplex non-viral delivery systems and does not disclose the use of viral vectors.

Additionally, pending claims 19-39 are not anticipated by the teaching of Chiou, et al because Chiou, et al. relates to the expression and secretion of the interferon polypeptide. Pending claims 19-39 relate to the expression of a intracellular interferon species. The teaching of Chiou does not teach or suggest that an intracellularly expressed interferon polypeptide would be efficacious. Consequently, Chiou, et al does not anticipate claims 19-39.

Conclusion:

Applicants believe that they have traversed all grounds of rejection set forth in the Office Action Dated October 4, 2000 in this application. Consequently, Applicants request that all grounds of rejection of the pending claims be withdrawn and that this application be passed to issuance without further delay.

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#6 attachment

Response to Office Action
USSN 09/353,423
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Exhibit A

p53 tumor suppressor gene therapy for cancer

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The last two decades have led to a greater understanding of the genetic basis of human malignancy. Although numerous genetic alterations have been detected in cancer, activation of oncogenes and inactivation of cell cycle regulators (e.g., tumor suppressor genes) are now known to play a critical role in the progression of the disease. Therapeutic strategies based on specific molecular alterations in cancer include reintroduction of wild-type tumor suppressor function to cells lacking the gene. p53 gene therapy provides an attractive strategy to test the potential clinical feasibility of this approach. Alterations in p53 function are present in approximately half of all malignancies, and expression of wild-type p53 can result in apoptosis in human tumor cells. This review summarizes current investigations with p53 gene therapy, highlighting the preclinical efforts with adenoviral, retroviral, and lipid-based gene delivery systems. A comprehensive review of the various clinical targets suggested for p53 gene therapy is presented together with challenges and prospects for future clinical investigation.

Key words: p53 gene therapy; p53 tumor suppressor gene; adenovirus; retrovirus; liposomes; gene therapy vectors; review article.

The p53 tumor suppressor is a 393-amino acid nuclear phosphoprotein that acts as a transcription factor to control the expression of proteins involved in the cell cycle.[1,2] In response to DNA damage, wild-type p53 accumulates in the nucleus and arrests the cell cycle via the cyclin-dependent kinase inhibitor p21WAF1/CIP1. Alternatively, p53 can induce apoptosis or programmed cell death through both transcription dependent (e.g., bax, Fas) and transcription-independent pathways. Because of these functions, p53 has been called the "guardian of the genome" and loss of p53 has been implicated in tumor progression. Functional inactivation of p53 can occur by several mechanisms including direct genetic mutation, binding to viral oncoproteins or cellular factors (e.g., mdm2), or alteration of the subcellular localization of the protein. Although p53 is not essential for normal development, p53 "knock-out" mice are susceptible to tumors early in life. Mutations in p53 have been reported in a majority of clinical cancers, and it has been estimated that p53 function is altered in half of all human malignancies. Non-random mutations in p53 have been reported in clinical specimens, and frequent mutations correspond to evolutionarily conserved regions of the molecule. Of particular significance, alterations in p53 are linked to poor prognosis, disease progression, and decreased sensitivity to chemotherapeutic agents. Detailed reviews on p53 function have appeared recently,[3–5] and the reader is directed to them for a more detailed description of p53 structure and function.

Consistent with the definition of a tumor suppressor gene, reintroduction of the wild-type p53 has been shown to be incompatible with the tumorigenic phenotype of many tumor cell lines. Early experimental work with neoplastic cells stably transduced with wild-type p53 demonstrated a suppression of cell growth, decrease in colony formation, and reduction in tumorigenicity in nude mice.[6,7] In addition, Shaw et al[8] provided evidence that re-expression of p53 in established tumors can induce apoptosis *in vivo*. More recent efforts, summarized below, have confirmed these initial findings with gene therapy vectors suitable for human clinical trials. Results from preclinical studies also suggest that non-transformed cells can tolerate exogenous expression of p53, providing a potential therapeutic index for the treatment of cancer.

GENE DELIVERY SYSTEMS

Clinical investigations using gene therapy have only recently been initiated, and many obstacles to efficient gene delivery have been identified. Successful gene therapy strategies will match a gene delivery system with a gene for a particular clinical application.[9] Although the p53 gene is altered in many human cancers, a single gene delivery system is not likely to be optimal for all indications. For example, intratumoral delivery to p53-altered head and neck tumors may require high local concentrations of a viral vector, whereas an alternative gene delivery system may be required to target blood-borne or metastatic disease. In addition, the requirement for integration of a transgene will govern the selection of a

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gene delivery system. Because overexpression of high levels of p53 can trigger apoptosis in tumor cells, gene delivery methods that result in transgene integration into the host genome are probably not necessary, or even desirable, for successful p53 gene therapy. The sections below summarize some of the advantages and disadvantages associated with the gene delivery systems currently in use. For detailed reviews on vector construction, the reader is referred to Jolly.[10]

Retroviruses

Replication-deficient retroviruses were the first viral vectors used in gene therapy and can accommodate approximately 8 kb of foreign DNA. Although these vectors have advantages for treating hereditary disorders, there are several disadvantages associated with retrovirus delivery of the p53 gene. Because retroviruses integrate genetic material into the host cell genome, both tumor and normal cells can be permanently modified. Although normal cells appear to tolerate the expression of exogenous wild-type p53, the potential for insertional mutagenesis raises safety concerns for retrovirus-based *in vivo* gene therapy. Retroviruses require cell division for efficient infection and transfer of new genetic information. Therefore, preferential transduction of rapidly dividing tumor cells can be expected with retroviruses. In addition, retroviral vectors unlike other viral gene delivery systems do not retain viral genes that may invoke an immune response. However, poor stability and low titers from current production processes are likely to limit the use of replication-deficient retroviruses to *ex vivo* gene therapy. Developments with retrovirus-based gene delivery have recently been reported,[11] but future improvements of these vectors are required to fully enable *in vivo* gene delivery of p53.

Adenoviruses

Alternative viral gene delivery systems have been explored for gene therapy, including those based on adenovirus, adeno-associated virus, herpes virus, and vaccinia virus. Among these, adenoviral vectors have received the greatest attention and have been used in clinical studies for cancer and cystic fibrosis.[9] Significant advantages of adenoviral vectors include 1) the ability to transduce both proliferating and quiescent cells, 2) a wide tissue tropism, and 3) the existence of efficient protocols for producing clinical-grade material at high concentrations. In contrast to retroviruses-mediated gene transfer, adenoviral DNA remains extrachromosomal, and integration into the host cell genome is not an obligate part of the viral life cycle. Consequently, transient transgene expression can be expected with replication-deficient, recombinant adenoviruses. Short term expression has obvious limitations for chronic gene replacement, but may be considered advantageous for the treatment of neoplasias. For example, transient overexpression of p53 in tumor tissue may be sufficient to activate the apoptotic pathway in neoplastic cells without the concerns associated with integration of genetic material into normal cells. Several investigators have reported the antitumor effects of adenoviruses encoding wild-type p53 in various preclinical models (see below).

Replication deficient adenoviruses have only recently been used in clinical studies. Severe adverse effects have not been reported following administration to patients with cystic fibrosis or cancer.[9] Interestingly, one published clinical study has reported the use of wild-type adenovirus when administered via the following routes: intratumoral, intra-arterial, and/or intravenous.[12] Thirty patients with advanced cervical cancer were dosed with no serious side-effects. Tumor necrosis was observed, but no pathological effects on vaginal, rectal, or bladder mucosa, or other tissues of the pelvis were found. There was no evidence of lesions in the central nervous system, liver, or other organs attributable to the adenovirus therapy.

Of particular concern with adenoviral vectors is the induction of a cellular and humoral immune response in treated patients. The nature and extent of the immune response to recombinant adenovirus has not been fully characterized in humans, but preclinical models suggest that neutralizing antibodies directed at the adenovirus capsid proteins may compromise gene transfer after repeat administration. A cytotoxic T-lymphocyte (CTL) response has also been reported after administration of recombinant adenoviruses. Although the relative contributions of the transgene and viral gene expression are currently unclear in this process, the host immune response leads to elimination of transduced cells. While the CTL response may provide an additional antitumor effect, the potential for toxicity to normal cells requires evaluation in both preclinical and clinical models.

Delivery of wild-type p53 via an adenoviral vector is likely to provide the first demonstration of effective p53 gene therapy in the clinic for a variety of human cancers. Because local administration is practical, initial clinical trials include intratumoral, intra-arterial (hepatic), intraperitoneal, and intracystic (bladder) delivery. Dosing strategies to overcome the expected immune response include transient immunosuppression to facilitate multiple administration as well as construction of "second generation" recombinant adenoviruses with limited expression of immunogenic, adenovirus late genes. Ongoing clinical trials will provide critical information on the dose and route dependence of the immune response to recombinant adenoviruses.

Nonviral gene delivery

Complexation of cationic lipids with DNA provides an alternative to viral gene delivery systems. Although less efficient, this lipid-based gene delivery system is likely to be less toxic and less immunogenic than strategies that use recombinant viruses. Both viral and nonviral gene delivery systems provide an opportunity to demonstrate the effects of p53 gene therapy on locoregional malignancies. However, delivery of the p53 gene to metastatic tumors via systemic administration will likely require

multiple administrations of a nonimmunogenic (i.e., nonviral) gene delivery system. The challenges associated with systemic delivery of macromolecules to tumors has been well documented.[13] Despite these obstacles, gene transfer to both normal and neoplastic tissue has been reported after lipid-based gene delivery.[14] Although the relative efficiency of transgene expression is low compared with viral vectors, systemic delivery of p53 has been shown to inhibit tumor growth in animal models. These studies suggest that the efficacy of p53 gene therapy may be due to broader mechanisms than originally suspected, i.e., inhibition of angiogenesis and other "bystander" effects (see below). Additional improvements in the efficiency of nonviral gene delivery systems are expected, and such improvements may enable systemic delivery that can be enhanced by tumor targeting.

CLINICAL TARGETS

Investigators have published studies on the effects of wild-type p53 gene therapy on more than 100 cell lines and tissues (Tables 1–3[15–65]). The general conclusion from these studies is that the introduction of wild-type p53 into neoplastic cells *in vitro* is lethal in most cells, if the cells are $p53^{mut}$ or $p53^{null}$. However, as Polyak et al[51] reported, some cell lines are only growth arrested. In most cases, $p53^{wt}$ neoplastic cells and normal cells were unaffected unless other factors influenced p53 function, such as human papillomavirus (HPV). Intratumoral dosing of $p53^{mut}$ or $p53^{null}$ tumor xenografts in immunocompromised mice resulted in tumor growth inhibition and often, tumor regression. In addition, two studies have demonstrated a reduction of $p53^{mut}$ mammary tumor metastases in the lungs after intravenous dosing with two different p53 vectors.[58,60] Dosing via the intrahepatic artery has also shown therapeutic benefits against $p53^{mut}$ hepatocellular carcinoma in Buffalo rats,[65] while dosing via the intrahepatic vein was not effective in SV40 Tag transgenic mice with liver tumors.[66]

Numerous investigators have demonstrated expression of wild-type p53 after introduction of DNA into cells using adenovirus, retrovirus, herpes simplex virus, liposomal, and plasmid vectors. All tissue-types tested so far have been permissive for exogenous p53 expression. Typical effects include changes in cell morphology, cell cycle arrest, and increased apoptosis or differentiation. Endogenous gene expression is often affected as well. Induction of p21 (WAF1/CIP1) has been reported after expression of exogenous p53 in prostate tumor cells,[19] ovarian tumor cells,[22] colorectal tumor cells,[22,51] head and neck tumor cells,[44] mammary tumor cells,[18,22] and mammary tumor xenografts.[60] Induction of MDM-2 has been reported in mammary[18] and medulloblastoma cells,[67] and *gadd45* expression was induced in ovarian tumor cells.[68]

Harris et al[16] correlated the percentage of tumor cells transduced by a β-gal adenovirus with the antiproliferative effects of a p53 adenovirus (Fig 1). In $p53^{null}$ and $p53^{mut}$ cells, they found a strong positive correlation between the degree of p53-induced growth inhibition and the rate of adenovirus transduction. In contrast, cell lines expressing normal levels of wild-type p53 were minimally affected by p53 transduction, independent of the adenovirus transduction rate.

Nielsen et al[15] found that the ability of a β-gal adenovirus to transduce three mammary tumor lines *in vitro* was predictive of the *in vivo* efficacy of a p53 adenovirus against tumor xenografts. At the same adenovirus concentrations, MDA-MB-468 cells had a slightly higher transduction rate than MDA-MB-231 cells, while MDA-MB-435 cells were resistant to adenovirus transduction. Intratumoral dosing of MDA-MB-468 and -231 xenografts resulted in significant growth inhibition and regression, while MDA-MB-435 xenografts were completely unaffected. By contrast, systemic dosing of MDA-MB-435 xenografts with a liposomal formulation of the p53 gene caused growth inhibition, regression, and reduced lung metastases.[60] These results strongly suggest that the lack of MDA-MB-435 tumor response in Nielsen et al[15] was not due to an inability of p53 to inhibit the growth and metastasis of MDA-MB-435 tumors, but rather, was due to the low adenovirus transduction efficiency of this cell line.

The α_v integrins have been implicated as cellular elements required for efficient internalization of type 2, 3, and 4 adenoviruses.[69–71] It is likely that α_v integrins perform the same role for type 5 adenovirus. Wickham et al[70] observed 5–10-fold higher internalization of a recombinant type 5 adenovirus in cells transfected with $\alpha_v\beta_5$ compared with cells lacking α_v expression or transfected with $\alpha_v\beta_3$. The human embryonic kidney 293 cells used for production of E1-deleted adenoviruses express $\alpha_v\beta_1$, but not $\alpha_v\beta_3$ integrins.[72] FACS analysis demonstrated that MDA-MB-231 and MDA-MB-435 cells both express roughly equivalent levels of all these integrin family molecules.[15] Therefore, the lack of adenovirus transduction in the MDA-MB-435 line is not due to a deficiency in integrin expression. It is possible that MDA-MB-435 cells are deficient in the cellular receptor required for adenovirus binding or that some other component required for viral binding, internalization, and gene expression is defective. Only recently, has a putative adenovirus cellular receptor (CAR1) been identified.[73] Future investigations with CAR1 should elucidate the resistance mechanism in MDA-MB-435 cells.

Prior to the identification of CAR1,[73] Seth et al[17] measured the number of adenovirus-binding sites on three mammary tumor cell lines (MDA-MB-231, -453, and MCF-7) and normal bone marrow cells. Mammary tumor cells had $1–2 \times 10^3$ high affinity and $5–8 \times 10^5$ low affinity binding sites for type 5 adenovirus. By contrast, adenovirus binding sites were undetectable on bone marrow cells. Binding site quantification was predictive of transduction rates by β-gal adenovirus with 100% of mammary cells transduced at 100 PFU/cell, but no bone marrow cells transduced at 500 PFU/cell. Colony formation by MDA-MB-231 cells was reduced 55% by 1 PFU/cell of p53 adenovirus and 100% by 10

Table 1. Efficacy of p53 Gene Therapy Against Tumor cells *in vitro*

Vector	Cell lines	ED_{50} values for effects of p53 gene therapy on cell proliferation (P) or colony formation (C)	Reference
Breast cancer			
Ad	MDA-MB-231, -468, -435	NS, NS, >50 CIU/cell (P)	15
Ad	MDA-MB-231, -468, SK-BR-3, BT-549, T-47D, HBL-100, MCF-7	12, 3, 16, 2, 3, 99, >100 CIU/cell (P)	16
Ad	MDA-MB-231, -453, MCF-7	1 PFU/cell (C), NS, NS; Apop	17
Ad	MDA-MB-231, -453, -157, MCF-7, 184B5, MCF10	0.4, 0.7, 0.3, 30, 5, 6 PFU/cell (P); Apop	18
Ad	MCF-7	No effect at 20 PFU/cell (P)	19
Ad	MCF-7	Decr at 200 PFU/cell (C)	20
Ad	MDA-MB-468	2 PFU/cell (P)	21
Ad	SkBr3, MCF-7	Decr (P); Apop; greater combined efficicacy with Dox, Mito, not Vincr (SkBr3)	22
Rtv	MDA-MB-468, BT549	Decr (C)	23
Ovarian cancer			
Ad	SK-OV-3, Caov-3, Caov-4, PA-1	24, >100, >100, >100 CIU/cell (P)	16
Ad	SK-OV-3	Decr (P), sensitized to radiation	24
Ad	SK-OV-3	110 CIU/cell (P), decr (C)	25
Ad	2774	10 μM (P)	26
Ad	SK-OV-3	10 PFU/cell (P)	21
Ad	SK-OV-3	Decr (P & C)	27
Ad	SK-OV-3	Decr (P)	22
Cervical cancer			
Ad	C33A, HT3, HeLa, C4-I, MS751, ME180, CaSki, SiHa	10, 63, 71, 97, 77, 138, 55, 37, PFU/cell (P); Apop	28
Ad	HeLa	80 CIU/cell (P)	16
Ad	HeLa	Decr (P)	29
Ad	HeLa	Decr (C)	20
Prostate cancer			
Ad	C4-2 (LNCaP), DU-145, PC-3	Decr (P)	30
Ad	LNCaP, DU-145, DuPro-1	Decr (P); Apop	19
Ad	Mouse 148-1PA	Decr (P)	31
Ad	Tsu-Pr1	Decr (P); Apop	32
Lung cancer			
Ad	H358, Calu-6, H661, H596, H23, H322, H460, MRC-9, A549, WI-38	2, 6, 7, 9, 3, 36, 81, 79, 79, 56 CIU/cell (P)	16
Ad	H358	0.17 PFU/cell (P)	18
Ad	H23	Sensitized to CDDP	33
Ad	H69, H596	4, 10 PFU/cell (P)	21
Ad	H226Br, H358, H322, H460	Decr (P)	34
Ad	H1299	Decr (P)	35
Ad	H358	Decr (P), sensitized to CDDP; Apop	36
Rtv	H226Br, H358	Decr (P)	37
Rtv	H358, H322, H460	Decr (P), decr (P), no effect	38
Rtv	H322, WT226	Decr (P), no effect (P)	39
Head and neck cancer			
Ad	Tu-138, MDA 686-LN	Decr (P)	40
Ad	TR146	Decr (P); Apop	41
Ad	Tu-138, Tu-177, MDA 686-LN, MDA 886	Decr (P)	40
Ad	Tu-138, Tu-177, MDA 686-LN, MDA 886	Decr (P)	42
Ad	Tu-138, MDA 686-LN	Decr (P); Apop	43
Ad	CNE-1, CNE-2Z	Decr (P and C); Apop	44
Nervous system cancer			
Ad	G55, G59, G112, G122, G124, U87 MG	6, 72, 1, 1, 1, 6 CIU/cell (P); Apop	45
Ad	G112, SK-N-MC, SN-N-SH	1, 2, 16 CIU/cell (P)	16
Ad	U-251 MG, T-98 G, U-87 MG, U-373 MG, U-138 MG, A-172, LG, EFC-2, D54 MG	Decr (P); Apop	46
Ad	Rat 9L	Decr (P)	47
Ad	T98G	Decr (P), sensitized to CDDP: Apop	33
Rtv	A673	Decr (C)	48

continued

Table 1. Continued

Vector	Cell lines	ED_{50} values for effects of p53 gene therapy on cell proliferation (P) or colony formation (C)	Reference
Bladder cancer			
Ad	HT-1376, 5637, J82, FHs 738B1	>100, 23, 40, >100 CIU/cell (P)	16
Colorectal cancer			
Ad	EB, Colo 320D, DLD-1, Colo 205, WiDr, SW480, SW837, RKO	45, 26, 12, >100, 47, 53, 52, >100 CIU/cell (P)	16
Ad	SW480	Decr (P), sensitized to radiation	49
Ad	DLD-1	7 CIU/cell (P)	21
Ad	SW620, KM12L4	Decr (P), Apop	50
Ad	SW480	Decr (P)	22
Ad	DLD-1, HCT116	Apop; Arrest	51
Liver cancer			
Ad	Hep 3B, HLE, HLF, SK-HEP-1, Hep G2	5, 1, 1, 96, 84 CIU/cell (P)	16
Ad	Hep 3B, Hep G2	7, 60 PFU/cell (P)	21
Skin cancer			
Ad	SK-MEL-24, Mouse B16	Decr (P); Apop	52
Muscle cancer			
Ad	A673, SK-UT-1	7, 5 CIU/cell (P)	16
Bone cancer			
Ad	Saos-2	2 CIU/cell (P)	16
Ad	Saos-2	Decr (P); Apop	53
Ad	Saos-2	Decr (P); Apop	54
Ad	Saos-2	1 PFU/cell (P)	21
Rtv	Saos-2	Decr (P, C)	6
Lymphomas/leukemias			
Vac	HL-60	Decr (P); incr Apop and differentiation	55
Ad	JB6	Decr (C)	20
Ad	K-562	>100 PFU/cell	21
Rtv	Be-13	Decr (C, P)	56
Normal tissue			
Ad	$CD34^-$ bone marrow	1000 PFU/cell (C)	17
Ad	Fibroblast	No effect (P)	42
Ad	Fibroblast	No effect at 20 PFU/cell (P)	19
Ad	Fibroblast	No effect (P)	26
Ad	Fibroblast	Variable decr (P)	27
Ad	Fibroblast	30–35% decr (P & C) at 50 PFU/cell	44
Ad	Mammary epithelium (NMEC)	100 PFU/cell (P)	18
Ad	Rat astrocyte	No effect (P)	47
Ad	Rat newborn neurons	Apop	57
Ad	Bronchial epithelium	No effect at 100 PFU/cell (P)	35

Ad = Adenovirus. Rtv = Retrovirus, Lip = Liposomal DNA, Vac = vaccinia virus. All cells are human unless otherwise indicated. P = cell proliferation inhibited. C = colony formation inhibited. ED_{50} = dose which caused a 50% reduction in P or C. Apop = apoptosis documented. NS = not studied. CDDP = cisplatin. Dox = Doxorubicin/Adriamycin. Mito = Mitomycin C.

PFU/cell. p53 adenovirus had no effect on colony formation by bone marrow cells at concentrations up to 100 PFU/cell. At a concentration of 1000 PFU/cell, bone marrow colonies were reduced by 50%, however at this very high adenovirus concentration cytotoxicity might be mediated by mechanisms other than p53 expression.

Viral oncoproteins, such as HPV E6, can inactivate wild-type p53 and hence, induce a mutant-p53 phenotype in cells lacking alterations in the p53 gene. HPV infections are especially prevalent in cervical tumors, with a growing number of reports in head and neck tumors.[74] Hamada et al[28] examined the efficacy of a p53 adenovirus in eight cervical cancer cell lines. Two of the lines expressed mutant p53, while the other six had wild-type p53 inactivated by HPV. Proliferation of all cell lines was inhibited by p53 adenovirus with a range of ED_{50} values from 9 to 149 PFU/cell. The p53 adenovirus induced apoptosis in infected cells, as well as, reversing tumorigenicity *in vivo*. In addition, p53 adenovirus treatment of established tumor xenografts from four cervical lines dramatically reduced tumor growth.

Combination therapy

Investigations into the efficacy of p53 gene therapy in combination with other therapeutics are only now start-

Table 2. p53 Protein Status of Cells Used in Gene Therapy Studies

Tissue	p53 status	Cell lines
Mammary	Mutant	BT-549, MDA-MB-231, MDA-MB-435, MDA-MB-453, MDA-MB-468, SK-BR-3, T-47D
	Null	MDA-MB-157
	Wild-type	184B5, HBL-100, MCF-7, MCF-10
Ovarian	Mutant	2774, Caov-4
	Null	SK-OV-3, Caov-3
	Wild-type	PA-1
Cervical	Mutant	C33A, HT3
	WT/HPV	C4-I, CaSki, HeLa, ME180, MS751, SiHa
Prostate	Mutant	DU-145
	Null	Mouse 148-1PA, Tsu-Pr1, PC-3
	Wild-type	PC-82, LNCaP (silent mutation)
Lung	Mutant	H23, H226Br, H322, H596, H661
	Null	Calu-6, H69, H358, H1299
	Wild-type	A549, MRC-9, WI-38, WT226, H460
Head/Neck	Mutant	TR146, Tu-138, Tu-177, CNE-1, CNE-2Z
	Null	SqCC/Y1
	Wild-type	MDA 686-LN, MDA 886,
Nervous Sys	Mutant	A172, Daoy, G59, G112, G122, G124, LG, 9L, T98G, U138MG, U251MG, U373MG, Del4A
	Null	A673, SK-N-MC
	Wild-type	EFC-2, D54 MG, G55, SN-N-SH, U87 MG
Bladder	Mutant	5637, J82, HT-1376
	Wild-type	FHs 738B1
	Unknown	Mouse MBT-2
Colorectal	Mutant	Colo 205, Colo 320D, DLD-1, SW480, SW620, SW837, WiDr, KM12L4
	Null	EB
	Wild-type	RKO, HCT116
Liver	Mutant	HLE, HLF, McA-RH7777
	Null	Hep 3B
	Wild-type	Hep G2, SK-HEP-1
Skin	Unknown	SK-MEL-24, Mouse B16
Muscle	Mutant	SK-UT-1
	Null	A673
Bone	Null	Saos-2
Leuk/Lymph	Null	K-562, U-937, HL-60, Be-13
	Unknown	JB6

Primary references for the p53 status of tumor cell lines can be downloaded at the Internet address fttp://ftp.ebi.ac.uk/pub/databases/p53 or can be found in the articles listed at the end of this review.

ing to appear. Fujiwara et al[36] demonstrated additive benefits in $p53^{null}$ H358 lung cancer when p53 gene therapy was combined with the DNA damaging agent, cisplatin. H358 cells cultured with cisplatin for 24 hours before transduction with p53 adenovirus had a significantly lower rate of proliferation than cells treated with either agent alone. When cells were transduced with p53 adenovirus 24 hours before exposure to cisplatin, there was a dose-dependent cisplatin effect. H358 cells or spheroids exposed to both agents exhibited greater apoptosis, as evidenced by DNA fragmentation. An additive efficacy of both agents, with enhanced apoptotic death, was also demonstrated *in vivo*. However, it should be noted that 1) the subcutaneous H358 xenografts were only 5 mm^3 at the beginning of the experiment and control tumors only reached a volume of 30 mm^3 on the last day of tumor measurements; 2) only 12 days elapsed from the start of dosing to the end of the two studies; and 3) the first cisplatin dose (3 mg/kg 3 ×) causes an average body weight loss of 26% in nude mice by day 7 and the 6 × dose would have been lethal (L.L.N., personal observation). Stronger evidence came from Nguyen et al.[61] In this study, $p53^{null}$ H1299 lung tumor xenografts were dosed with intraperitoneal cisplatin before, concurrent with, or after intratumoral p53 adenovirus. The most effective dosing regime was 5 mg/kg cisplatin given 2 days before three doses of 5×10^9 viral particles/day of p53 adenovirus, with the adenovirus doses administered 2 days apart. A second cycle of therapy produced increased efficacy over a single cycle.

Gjerset et al[33] demonstrated increased sensitivity to cisplatin cytotoxicity in $p53^{mut}$ T98G glioblastoma and $p53^{mut}$ H23 small cell lung carcinoma cells transduced with p53 expression vectors 1 or 2 days before cisplatin exposure. Cell death mediated by apoptosis was significantly increased versus p53-transduced cells, when T98G cells were transduced by 100 PFU/cell of p53 adenovirus 2 days before exposure to 30 μM cisplatin. Additive efficacy was also seen for p53 and γ-irradiation. Yang et al[75] used $p53^{mut}$ SW480 colorectal tumor cells transfected with an IPTG-inducible p53 plasmid construct to evaluate the combined efficacies of p53 with 5-fluorouracil (5-FU; 0–20 μM), p53 with topotecan (0–10 μM), and p53 with γ-irradiation (0–400 cGy for 1 hour). All three agents displayed dose-dependent effects on cell cytotoxicity which were enhanced by concurrent expression of wild-type p53. DNA fragmentation was elevated in cells exposed to both p53 and 5-FU. Furthermore, the potentiation of 5-FU cytotoxicity by p53 was greatest when cells were exposed to both agents simultaneously. Blagosklonny and El-Deiry[22] reported increased cell killing in $p53^{mut}$ SkBr3 mammary tumor cells when transduction with p53 Ad was followed 8 hours later by doxorubicin or mitomycin C, but not by vincristine. Greater combined efficacy was not observed in $p53^{wt}$ MCF-7 mammary tumor cells for any of the three drugs.

Additional studies on the ability of wild-type p53 to sensitize tumor cells to irradiation have been reported for colorectal and ovarian tumor cells.[24,49] SW620 colorectal tumor cells ($p53^{mut}$) were transduced with 50 PFU/cell p53 adenovirus 48 hours before irradiation with 2 or 4 Gy.[49] Cell survival was reduced by 50–66% compared to mock- or vector-infected irradiated cells, and this reduction was mediated by apoptotic cell death. Efficacy was also highest in SW620 xenografts pretreated with three consecutive doses of p53 adenovirus before irradiation with 5 Gy. Again, apoptosis was most evident in tumors treated with both agents. Similar, although not as dramatic, results have been reported for $p53^{null}$

Table 3. Efficacy of p53 Gene Therapy Against Tumor Xenografts

Cell line	Vector	Route	Dose *in vivo*	Efficacy	Reference
Breast cancer					
MDA-MB-231	Ad	T/P	2×10^8 CIU, 10×	95% growth inhibition, 30% tumor-free, Apop	58
MDA-MB-468	Ad	T/P	2×10^8 CIU, 10×	80% growth inhibition, 10% tumor-free, Apop	58
MDA-MB-435	Ad	T/P	2×10^8 CIU, 10×	No efficacy	58
MDA-MB-231	Ad	T/P	$2–4 \times 10^8$ CIU, 10×	79–86% growth inhibition; 60–80% decr lung metastases	15
MDA-MB-231	Ad	i.v.	4×10^8 CIU, 5×	71% decr no. lung metas, decr metastases size	15
MDA-MB-435	Lip	i.v.	16/12 μg DNA, 2×	75% growth inhib	59
MDA-MB-435	Lip	i.v.	35 μg DNA, 6×	Growth inhib, 53% regressed, 67% lung metastasis-free	60
MCF-7	Lip	i.v.	16/12 μg DNA, 3×	40% growth inhibition	59
Ovarian cancer					
SK-OV-3	Ad	T/P	1×10^8 PFU, 1×	Sensitized to irradiation	24
SK-OV-3	Ad	e.v., i.p.	2×10^8 CIU, 6×	Incr survival; marginal incr survival	25
			2×10^9 CIU, 6×	Marginal incr survival	
Cervical cancer					
C33A	Ad	e.v., T/P	5×10^9 PFU, 6×	100% tumor suppression; 96% growth inhib & 29% tumor-free	28
HT3	Ad	e.v., T/P	5×10^9 PFU, 6×	100% tumor suppression; 96% growth inhib & 71% tumor-free	28
HeLa	Ad	e.v.	NA	100% tumor suppression	28
MS751	Ad	e.v., T/P	5×10^9 PFU, 6×	100% tumor suppression; 88% growth inhib & 14% tumor-free	28
SiHa	Ad	e.v., T/P	5×10^9 PFU, 1×, 3×, 6×	100% tumor suppression; 62% growth inhib, 92% growth inhib; 95% growth inhib & 20% tumor-free	28
Prostate cancer					
C4-2 (LNCaP)	Ad	T/P	1×10^9 PFU, 6×, 8×	Growth inhib, 88% tumor-free; Apop	30
DU-145	Ad	e.v.	NA	100% tumor suppression	30
PC-3	Ad	e.v.	NA	100% tumor suppression	30
Mouse 148-1PA	Ad	T/P	5×10^9 PFU, 1×	21% growth inhibition	31
Tsu-Pr1	Ad	e.v.	NA	90% tumor suppression	32
Lung cancer					
H1299	Ad	T/P	5×10^9 PN/cell,	Incr efficacy in combination with CDDP (1, 2)	61
H69	Ad	T/P	2×10^9 PFU, 1×, 8×	Incr survival	21
H226Br	Ad	i.t.	5×10^7 PFU, 2×	73% growth inhibition, 75% tumor-free vs 20–30% of controls	34
H358	Ad	T/P	2×10^7 PFU, 3×	Growth inhibition; Additive efficacy with CDDP (3)	36
H226Br	Rtv	i.t.	8×10^5 PFU, 3×	64% growth inhib, 63% tumor-free vs. 25% of controls	37
Head and neck cancer					
Tu-138	Ad	T/P	1×10^8 PFU, 1×	100% tumor suppression	42
Tu-177	Ad	T/P	1×10^8 PFU, 1×	100% tumor suppression	42
MDA 686-LN	Ad	T/P	1×10^8 PFU, 1×	100% tumor suppression	42
MDA 886	Ad	T/P	1×10^8 PFU, 1×	67% tumor suppression	42
MDA 686-LN	Ad	T/P	1×10^7 PFU, 1×	Apoptosis in tumors	43
Tu-138	Ad	T/P	1×10^8 PFU, 1×	97% growth inhibition	62
Tu-177	Ad	T/P	1×10^8 PFU, 1×	98% growth inhibition	62
Nervous system cancer					
G122	Ad	e.v, T/P	7×10^8 CIU, 3×	100% tumor suppression	45
Rat 9L	Ad	T/P	1×10^7 PFU, 1×	40% growth inhibition	47
A673	Rtv	e.v.	NA	Tumor suppression	48
Bladder cancer					
Mouse MBT-2	Ad	i.s., i.p.	1.5×10^9 PFU, 1×	No efficacy	63
Colorectal cancer					
DLD-1	Ad	T/P	1×10^9 CIU, 5×	Growth inhibition, incr survival	16
SW620	Ad	T/P	2.5×10^9 PFU, 3×	Growth inhibition, incr apoptosis after irradiation	49
SW620, KM12L4	Ad	T/P	3.3×10^9 PFU, 3×	Growth inhibition, incr apoptosis	50
Liver cancer					
McA-RH7777	Ad	IHA	???	Growth inhibition	64
Skin cancer					
SK-MEL-24	Ad	T/P	2×10^9 PFU, 1X	Growth inhibition	52
Mouse B16	Ad	T/P	2×10^9 PFU, 1X	Growth inhibition	52

continued

Table 3. Continued

Cell lines	Vector	Route	Dose *in vivo*	Efficacy	Reference
Bone cancer					
Saos-2	Ad	e.v.	NA	100% tumor suppression	21
Saos-2	Rtv	e.v.	NA	100% tumor suppression	6
Normal tissue					
Rat liver	Ad	PV	5×10^9 PFU, 1X	No effect on liver regeneration after hepatectomy	65

Ad = adenovirus, Lip = liposomal DNA, Rtv = retrovirus, T/P = intra-/peritumoral, i.v. = intravenous, i.p. = intraperitoneal, i.t. = intratracheal, IHA = intrahepatic artery, PV = hepatic portal vein, e.v. = *ex vivo*, i.s. = intravesicular, NA = not applicable, CDDP = cisplatin at: 1) 5 mg/kg, i.p., 1 ×; 2) 1.67 mg/kg, 3 ×; 3) 3 mg/kg, i.p., 3 times. Apop = apoptosis-documented. All cells are human unless otherwise indicated.

SK-OV-3 ovarian tumor cells.[24] Cells transduced with p53 adenovirus and subsequently irradiated with 2 or 4 Gy had approximately 8–30% lower survival than mock- or vector-infected irradiated cells. Subcutaneous tumor xenografts were treated once with p53 adenovirus or the appropriate controls, then irradiated with 4 Gy/day on 3 consecutive days. This dosing regime was repeated 1 week later. Combination therapy with p53 and irradiation had significantly increased efficacy against tumor xenografts and cured 45% of the mice.

The preliminary conclusion, which can be gleaned from these seven studies,[22,24,33,36,49,61,75] is that p53 gene therapy combined with DNA-damaging agents has additional efficacy over p53 gene therapy alone. Further, no observations of antagonistic interactions between p53 gene therapy and more traditional anticancer therapeutic agents have been reported. In particular, cisplatin pretreatment might sensitize tumors to subsequent p53 gene therapy. The converse situation, p53 pretreatment of tumors, has not proven as effective in sensitizing tumors to cisplatin. However, this result may be due to physical factors and not a true deficiency in p53 effects. When mice are given intraperitoneal doses of cisplatin, the drug reaches most, if not all, tumor cells via the blood supply. On the other hand, the *in vivo* experiments reported to date have used an adenovirus vector and direct injection into the xenograft and surrounding tissues to deliver the p53 gene. The architecture of this tumor system limits diffusion of the adenovirus, and thereby, limits the number of infectable target cells which come in contact with the adenovirus. In very small tumors, a sizeable fraction of the tumor cells may be transduced by one dose of p53 adenovirus, but this situation is the exception rather than the rule. This was clearly demonstrated in Nielsen et al,[15] where fractionated doses of p53 adenovirus had increased antitumor efficacy over fewer, higher doses.

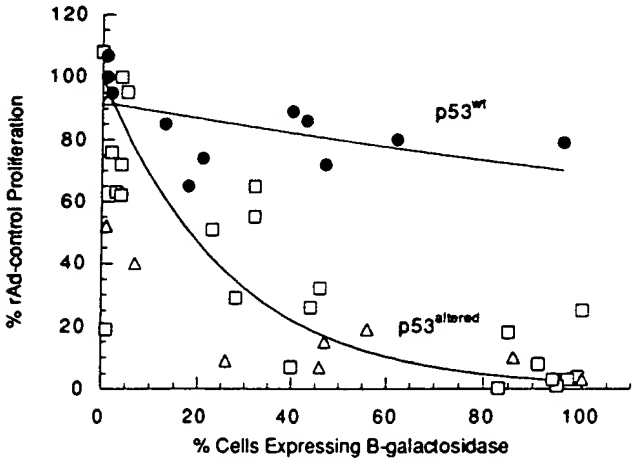

Figure 1. p53-specific growth inhibition as a function of adenovirus transduction. p53-specific antiproliferative effects of p53 adenovirus were measured at 30 CIU/cell in a 72 hours [^{3}H]thymidine incorporation assay and normalized for the effects of an adenovirus vector control. Adenovirus transduction was measured as the percentage of cells expressing β-galactosidase 24 hours after infection with a β-gal adenovirus. Open symbols represent individual p53-altered cell lines (squares, $p53^{mut}$; triangles, $p53^{null}$). Closed symbols represent cell lines expressing endogenous wild-type p53. Data are presented as means of replicate experiments. (Reprinted from Ref. 16).

In a eighth study on combination therapy, cell differentiation, not cell death was the end result. Ehringer et al[76] transfected $p53^{null}$ U-937 leukemia cells with a temperature-sensitive murine p53 mutant which converted to wild-type conformation at 32°C. Expression of wild-type, but not mutant, p53 slowed cell proliferation, caused cells to accumulate in the G1 phase of the cell cycle, and induced apoptosis. Somewhat paradoxically, expression of wild-type p53 sensitized cells to differentiation mediated by vitamin D3 and this effect "overrode" the apoptosis pathway.

Clinical efficacy

The potential for toxicity in normal tissue, caused by the expression of exogenous p53, is an issue of concern for any clinical protocol. Preclinical data are encouraging on this issue. Investigators have observed little or no detrimental effects on normal fibroblasts, bronchial epithelium, mammary epithelial cells, bone marrow cells, rat astrocyte cells, and rat liver at concentrations of p53 which are highly effective at killing neoplastic cells.[17–19,26,27,35,42,47]. The most common route of p53 administration to tumor xenografts has been through intra- and peritumoral injection. No gross necrosis or other abnormalities of tissues surrounding injection sites has been reported in the literature or observed in our laboratories (L.N. and D.M.). One cautionary report has been published.[57] The

investigators transduced newborn Sprague–Dawley rat neurons with recombinant adenoviruses *in vitro* and found dose-dependent p53-specific toxicity, in addition to the vector-specific toxicity discussed below.

To date, only one clinical study has been published in which vector transduction of target tissues was confirmed. Roth et al[77] transduced lung tumors using a p53 retrovirus introduced into patients via fiberoptic bronchoscope or percutaneous needle with radiologic guidance. Nine male patients with a history of primary non-small cell lung carcinoma (NSCLC) and recurrent or metastastic tumors were enrolled in the phase I study. All nine patients had mutations in the p53 gene. Vector sequences were detected in eight of the treated tumors. In addition, six out of seven evaluated tumors showed evidence of increased apoptosis. Tumors regressed in three of the seven patients and no toxicity due to p53 therapy was observed. The results of this promising phase I human trial support cautious optimism for the future of p53 gene therapy of cancer.

FUTURE CHALLENGES

Vector toxicology

The most significant elements in the emerging toxicological profile for recombinant adenoviruses are the localized inflammatory response at the site of administration and the interference with normal hepatocyte functioning caused by high intravascular concentrations of virus. Zhang et al[35] reported on the toxicology of E1-deleted p53 adenovirus in mouse lungs. Intratracheal adenovirus at 10^7 to 10^{10} PFU/mouse was administered to Balb/c mice, and lungs were harvested 1, 3, 6, and 12 days after inoculation. No pathological changes were observed at the 10^7 and 10^8 PFU dose levels. However, at the 10^9 and 10^{10} PFU doses there was a mild inflammation characterized by perivascular and peribronchial infiltration of mononuclear cells.

At least three groups have reported changes in hepatocyte function at very high doses of E1-deleted adenovirus.[78–80] Cultured mouse hepatocytes were 100% transduced by recombinant adenovirus at 100 PFU/cell without any toxic effects, however at virus concentrations $\geq$ 1000 PFU/cell cytotoxicity was observed.[78] C57Bl/6 mice dosed with 1×10^{10} viral particles via the hepatic portal vein did not exhibit toxic effects. However, infusion of 7×10^{10} viral particles was lethal in most of the mice, due to liver necrosis. Drazan et al[79] studied liver function in male Brown Norway rats after *ex vivo* infusion of β-gal adenovirus or empty vector via the hepatic portal vein and subsequent liver transplantation into syngeneic hosts. Transduction with 50 PFU/cell β-gal adenovirus resulted in ascites and lethality due to liver necrosis in three out of four rats. Liver necrosis was absent in livers infused with vehicle or empty vector. Yang et al[80] infused 1×10^{10} PFU retrograde into the biliary tracts of female CBA and athymic nude mice. CBA mice infused with a β-gal adenovirus developed liver pathology characterized by ballooning degeneration of hepatocytes and cell death, followed by increased hepatic mitoses and some lymphocytic infiltration. Nude mice had similar hepatocyte abnormalities, but no lymphocyte infiltration and hepatic mitoses.

At least one group has reported on the effects of intravenous p53 liposomes on organ histopathology.[59] The first p53 liposome dose contained 16 μg of DNA with 400 nmol of liposome (= 1:1 ratio of 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine and dioleoyl phosphatidylethanolamine). Ten days later a second injection of 12 μg of DNA with 400 nmol of liposome was given. Athymic nude mouse organs were harvested 14 days after the last injection. No pathological changes were observed in heart, lung, liver, pancreas, spleen, kidney, intestine, or skin. Also, blood chemistry was unchanged by p53 liposomal treatment.

Immune response to adenoviruses

Prevailing theory holds that adenovirus infection generates a rapid inflammatory and cytolytic response mediated by cytotoxic T cells in hosts with fully functional immune systems.[81] This T-cell response is stimulated by adenovirus antigens produced in host cells and presented in conjunction with major histocompatibility complex moieties on the cell surface. Neutralizing antibodies specific for cells transduced by adenovirus are produced later in the immune response and are believed responsible for the reduced ability to re-infect host cells with adenovirus after initial inoculations.

One proposed solution to the adenovirus immune system problem is to create vectors in which most of the late viral genes are deleted. A cautionary note for this strategy can be found in Adesanya et al,[82] where injection of either bioactive or UV-inactivated adenoviruses into rat salivary gland caused a sharp reduction in saliva production, in other words, inflammatory cell infiltration and tissue damage. This occurred despite the lack of gene transcription from the UV-irradiated virus. Adenoviral and liposomal vectors both cause some tumor growth inhibition without p53 expression in mouse xenograft models. The mechanism of this antitumor effect is unclear at present. It may be partially mediated by nonspecific immune cells such as NK cells,[54] however other mechanisms are also likely to play a role.

Bystander effects

There is some evidence that p53 might exert some of its antitumor activity through inhibition of angiogenesis.[59,83] $p53^{null}$ fibroblasts from Li-Fraumeni patients secreted reduced levels of thrombospondin-1, an angiogenesis inhibitor, compared to early passage $p53^{wt}$ fibroblasts.[78] An anti-thrombospondin-1 antibody restored the migration of bFGF-stimulated capillary endothelial cells which was induced by $p53^{wt}$ fibroblast-conditioned medium, implying a role for thrombospondin-1 in angiogenesis suppression. Restoration of wild-type p53 function in $p53^{null}$ fibroblasts resulted in higher levels of thrombospondin-1 and lower angiogenic activity in conditioned medium. Other evidence for a role of p53 in

suppressing angiogenesis was reported by Xu et al.[59] MDA-MB-435 mammary tumor xenografts in nude mice showed significant growth inhibition and reduced blood vessel density when mice were dosed with intravenous p53 liposomes. This result is especially surprising given the low tumor transduction rate of 5% in this experiment.

CONCLUDING REMARKS

The preclinical studies reviewed above have clearly demonstrated the feasibility of p53 gene therapy for cancer in a variety of models. Expression of p53 in cancer cells lacking this tumor suppressor can lead to cell apoptosis or cycle arrest, and delivery of p53 may also inhibit the angiogenesis required for tumor growth. Although retroviral vectors were used initially to demonstrate the utility of p53 reintroduction, alternative delivery strategies are required for successful p53 gene therapy in the clinical setting. Adenoviral vectors provide an efficient method for locoregional delivery and transient overexpression of p53, and this strategy may enable effective p53 gene therapy for the treatment of certain malignancies. Ongoing clinical investigations will provide critical information on the safety and efficacy of this approach. However, significant advances in current gene delivery technology are needed to increase the efficiency of gene transfer to metastatic disease.

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Exhibit B

→SCH58500 CLINICAL PUB

CHAPTER 32

Opportunities for p53 Tumor Suppressor Gene Therapy in Ovarian and Other Peritoneal Cancers

Loretta L. Nielsen, Mark Pegram, Beth Karlan, John Elkas, and Jo Ann Horowitz

Schering-Plough Research Institute is currently sponsoring phase I/II clinical trials of adenovirus-mediated p53 gene therapy for cancer in several countries. The drug used in these clinical trials (Ad p53; ACN53; SCH58500) consists of a replication-deficient, type 5 adenovirus vector expressing human p53 tumor suppressor gene under the control of the cytomegalovirus promoter.[1] Clinical targets for initial trials with p53 gene therapy were chosen on the basis of frequency of p53 mutation and pattern of spread for specific tumors which would make them amenable to local or regional therapy and subsequent biopsy. The incidence of p53 mutations for most tumors is dependent on stage of the disease. In general, early stage disease has a much lower incidence of p53 mutation, with more advanced and invasive cancer having a higher incidence of mutation. The natural history of ovarian cancer results in 75% of patients having cancer spread beyond the ovary and 60% beyond the pelvis at diagnosis. Neoplastic cell dissemination within the peritoneal cavity is the most common pathway for progression in patients with advanced disease. Five year survival for patients with regional disease at diagnosis is 55%. Preexisting anti-adenoviral antibodies and the need to achieve an effective concentration of Ad p53 suggest this drug will be most effective when administered regionally. Ovarian cancer limited to the abdominal cavity, with small volume disease either naturally or optimally debulked, seems ideal for regional p53 gene therapy.

Preclinical Pharmacology: Intraperitoneal Administration of p53 Adenovirus

Many studies have examined the in vivo efficacy of Ad p53 in preclinical models when adminstered intratumorally or intravenously;[2] however, published information does little to guide clinicians in the design of intraperitoneal (i.p.) dosing trials for ovarian cancer. To this end, we examined several parameters with special significance for i.p. adminstration of Ad p53. First, cell proliferation was measured in two ovarian tumor lines (SK-OV-3, OVCAR-3) and one prostate tumor line (DU-145) after in vitro treatment with Ad p53. SK-OV-3 cells are $p53^{null}$, while OVCAR-3 and DU-145 cells express mutant p53. The ED_{50} values (ciu/cell required for a 50% reduction in cell number) were approximately 30 for SK-OV-3 cells, 2 for OVCAR-3 cells, and 5 for DU-145 cells. The effects of dosing volume

Adenoviruses: Basic Biology to Gene Therapy, edited by Prem Seth.

id frequency were next examined, using the SK-OV-3 tumor xenograft model in SCID ide.[3] When the effect of vehicle volume on antitumor efficacy was examined, all mice :ated with Ad-p53 had reduced tumor burden compared to controls. Vehicle volumes :tween 0.2 and 1 ml were equally effective, and all were more effective than a vehicle volume 0.1 ml. However, reduced efficacy was observed when a 1.5 ml vehicle volume was lministered. These results are somewhat puzzling, but might be explained by considering e fact that 1.5 ml is a large volume of liquid to inject into the peritoneal cavity of a 20 am mouse. It appears that the peritoneal tumor was not exposed to the entire dose of l-p53 when this volume was used. Therefore, hydrostatic pressure may have caused leakage ck out of the injection site and/or into the systemic circulation prior to adenovirus ınsduction of peritoneal tumor cells. Volume considerations have also determined the aximum viral dose available for phase II clinical trials in human patients, given the lack of ıse-limiting toxicities observed in our phase I trials (see clinical section in this chapter). ext, the effect of dosing frequency was examined in the same SK-OV-3 model. Fraction- ed doses of Ad p53 had greatly enhanced efficacy compared to fewer, bolus injections. By ntrast, bolus and fractionated doses were equally effective in the DU-145 model. This ggests that the optimal dosing regime in mouse models will be dependent on specific ıaracteristics associated with each tumor cell line. Multiple variables may be involved; erefore, extensive studies with many different i.p. models will be needed to isolate the itical factors. For example, i.p. DU-145 tumors grow faster than i.p. SK-OV-3 tumors in CID mide, but SK-OV-3 tumors kill the mice faster at tumor burdens between 1 and 2 g. contrast, DU-145 tumors can reach tumor burdens of 5-6 g before they kill their host. ımor burden at the start of dosing is also important, if the "peeling-the-onion" theory of ug action is correct. According to this hypothesis, the particulate drug (Ad p53) has limited ility to penetrate outer cell layers in a peritoneal tumor. Therefore, each dose, no matter w concentrated, can only kill the outer tumor layers. Once those cells die and disintegrate, e next dose of drug can access another discrete layer of tumor. Preliminary studies using ımunohistochemistry and Laser Scanning Cytometry to assess adenovirus transduction, ınsgene expression, and p53-induced apoptosis support this theory.[4] However, the biology is obably more complex than this simple model. For example, Nielsen et al[3] have shown that combinant adenoviruses can be absorbed from the peritoneal cavity into the systemic rculation when delivered at high concentrations for several consecutive days.

d-p53 Gene Therapy Combined with Chemotherapy

Cancers containing nonfunctional p53 tumor suppressor protein are generally less nsitive to chemotherapy.[5] Many anticancer agents induce apoptosis via p53-dependent isplatin, doxorubicin, 5-fluorouracil) or p53-independent (paclitaxel) pathways.[5-7] ıerefore, the introduction of wild type p53 into cells with nonfunctional p53 protein ıould enhance their sensitivity to most chemotherapeutic drugs. Although systemic elivery of chemotherapy is commonly employed in the treatment of metastatic disease, e results are frequently disappointing. Enhancement of its efficacy may result in more fective treatment outcomes.

Ad p53 combined with cisplatin, doxorubicin, 5-fluorouracil, methotrexate, etoposide, paclitaxel (taxol) inhibited cell proliferation more effectively than chemotherapy alone $p53^{null}$ SCC-9 head and neck, $p53^{null}$ SCC-15 head and neck, $p53^{null}$ SCC-25 head and ck, $p53^{null}$ SK-OV-3 ovarian, $p53^{mut}$ OVCAR-3 ovarian, $p53^{mut}$ DU-145 prostate, $p53^{mut}$ DA-MB-468 breast and $p53^{mut}$ MDA-MB-231 breast tumor cells.[8,9] Responses were dependent of the type of p53 gene mutation in the cells, and cells expressing mutant p53 otein were indistinguisable from $p53^{null}$ cells. Also, no obvious schedule dependence was ıserved.

In addition to the reasons mentioned above, further rationale for combining p53 gene therapy with chemotherapy in the clinical setting are:

1. Combinations of agents with different toxicological profiles can result in increased efficacy without increased overall toxicity to the patient;
2. Combinations of therapeutic agents may thwart the development of resistance to single agents;
3. Combinations of therapeutic agents may offer a solution to the problem of heterogeneous tumor cell populations with different drug sensitivity profiles;
4. Combinations of therapeutic agents allow physicians to take advantage of possible synergies between drugs, resulting in increased anticancer efficacy in patients.[10] Synergy (or antagonism) between two chemical agents is an empirical phenomenon, in which the observed effect of the combination is more (or less) than what would be predicted from the effects of each agent working alone. Although mathematical synergy cannot be directly proven in the clinic, it does predict in vivo synergy when the two therapeutics are combined. By contrast, overt antagonism warns of future clinical problems.

Sophisticated statistical modeling techniques were used to evaluate the pres[redacted] of synergistic, additive, or antagonistic efficacy between Ad p53 and paclitaxel in a panel of human tumor cell lines.[8] Tumor cells with altered p53 were treated with paclitaxel 24 hours before Ad p53 or treated with both agents simultaneously. Paclitaxel had synergistic or additive efficacy in combination with Ad p53, independent of whether the cells expressed mutant p53 protein or no p53 protein at all. Most importantly, antagonism between the two drugs was never observed. Paclitaxel increased the number of cells transduced by recombinant adenovirus 3-35% in a dose-dependent manner at paclitaxel concentrations up to 12 nM. This is one possible mechanism to explain the observation of drug synergy. In other words, more tumor cells were infected with Ad p53 and exposed to high levels of wild type p53 protein when paclitaxel "sensitized" them to transduction by recombinant adenovirus. Of particular note, the concentrations of paclitaxel responsible for increased adenovirus transduction were lower than the concentrations required for microtubule condensation. Also, the rate of change in the number of cells transduced by adenovirus appeared to be independent of paclitaxel-induced cell death.

The antitumor effects of combination therapy with Ad p53 and paclitaxel were also evaluated in tumor xenograft models in vivo. It has been well documented that Ad p53 is a drug with antitumor efficacy attributable to both the p53 tumor suppressor gene and the adenovirus delivery vector.[2] The in vivo experiments were designed to mimic the [redacted]ical situation, in which efficacy of the Ad p53 drug (with or without chemotherapy) will be compared to clinical outcome with traditional chemotherapy. In this situation, it is unethical and prohibitively expensive to include study arms for an empty adenovirus vector. In the intraperitoneal SK-OV-3 model of ovarian cancer, a dose of Ad p53 which had relatively minimal antitumor effect by itself had significantly enhanced efficacy when combined with paclitaxel. Similar results were observed using a higher dose of Ad p53. Paclitaxel also enhanced the antitumor efficacy of Ad p53 in the DU-145 prostate, MDA-MB-468 breast, and MDA-MB-231 breast cancer xenograft models. In summary, adenovirus-mediated p53 gene therapy for cancer shows enhanced therapeutic benefit when combined with paclitaxel. The ability of paclitaxel to increase adenovirus transduction rates in tumor cells could explain part or all of the observed enhancement.

Greater anticancer efficacy was also demonstrated with other chemotherapy drugs in tumor xenograft models in vivo.[9] These data support the combination of p53 gene therapy with chemotherapy in clinical trials. Of particular significance, there was enhanced efficacy using the three drug combination of Ad p53, cisplatin, and paclitaxel in the SK-OV-3 ovarian

ıor model. Clinical trials combining Ad p53 with chemotherapy in liver, lung, and ovarian cers are currently underway.

mor/Host Factors Predictive of Response to p53 Adenovirus

Nonfunctional p53 in and of itself may not be a sufficient predictive factor of clinical ɔonse, because other tumor and host factors may interfere with adenovirus-mediated e delivery. To cite an analagous situation, chondroitin sulfates in malignant pleural ısions were recently shown to inhibit gene transfer by retroviral vectors.[11] Analyses of mechanism of this effect indicated that interaction of chondroitin sulfates with retroviral .or in solution was responsible for inhibition. Further, pretreatment of pleural fluid with ndroitinases abolished the inhibitory activity. To investigate whether there are host factors ch might inhibit adenoviral vectors, we analyzed the effect of malignant ascites fluid ıined from patients with ovarian carcinoma on transduction efficiency of Adβ-Gal into)A-MB-231, p53^{mut}, breast carcinoma cells. The results demonstrate that a 50/50 mixture nalignant ascites and cell culture medium inhibited β-Gal transduction efficiency by 100% in 17 of 18 samples analyzed (Fig. 32.1). In addition, this inhibitory factor resides ıe soluble, rather than the cellular, fraction of the ascites. Conditioned media from tumor cultures derived from the same patients were not able to block Adβ-Gal transduction, gesting that this inhibitory factor is derived from normal tissues rather than from ignant cells. Further isolation and characterization studies are ongoing. This data strongly gests that removal of malignant ascites from patients prior to intraperitoneal treatment ı adenovirus-based gene therapies could facilitate transgene delivery to tumor cells in).

We are also examining the influence of factors which may be predictive of response to ɔ53. Preliminary data suggests that the transduction efficiency of Adβ-Gal in a panel of ıary ovarian tumor cell cultures is predictive of in vitro tumor cell response to Ad p53 . 32.2). For example, the primary ovarian carcinoma cell cultures CSOC 823c and 5,6 have mutations of p53 (determined by immunohistochemistry) and yet are not vth suppressed by Ad-p53. This lack of p53 activity correlates with low transduction iency by Adβ-Gal. We are currently analyzing expression levels of the common receptor oxsackie B and adenoviruses 2 and 5 (CAR)[12] in a large panel of ovarian tumor-derived cultures to test the hypothesis that CAR is necessary for efficient transduction by -Gal. The predicted amino acid sequence from HeLa cell-derived CAR cDNA indicates CAR is a 365 amino acid transmembrane protein with two extracellular ıunoglobulin-like domains. Though the cellular function of CAR remains unknown, ɔfection and expression of CAR in CHO cells is sufficient for adenovirus-mediated transfer by Adβ-Gal. It is our hope that investigation of tumor cell factors, such as , and host factors which influence adenovirus-based gene delivery systems will allow ɔptimization of patient selection and improved therapeutic potential for Ad p53.

ıical Results: Intraperitoneal Administration of p53 Adenovirus

A multinational phase I clinical trial was initiated in January 1997. The original intent is trial was to determine drug safety and transgene (p53) expression after intraperitoneal inistration of Ad p53. The drug was administered in cohorts of three patients in a rising fashion. Patients with intraperitoneal disease amenable to biopsy or cellular cytospin, r by laparoscopy or paracentesis, were enrolled. All patients had anti-Ad antibodies to gene therapy, a good performance status, acceptable baseline laboratory values, and ctive wild type adenovirus infection, as confirmed by ELISA. The initial dose was 7.5 x viral particles (pn) diluted in 1 liter immediately prior to intraperitoneal injection. equent cohorts of three patient were enrolled at the following dose levels: 7.5 x 10^{11} pn,

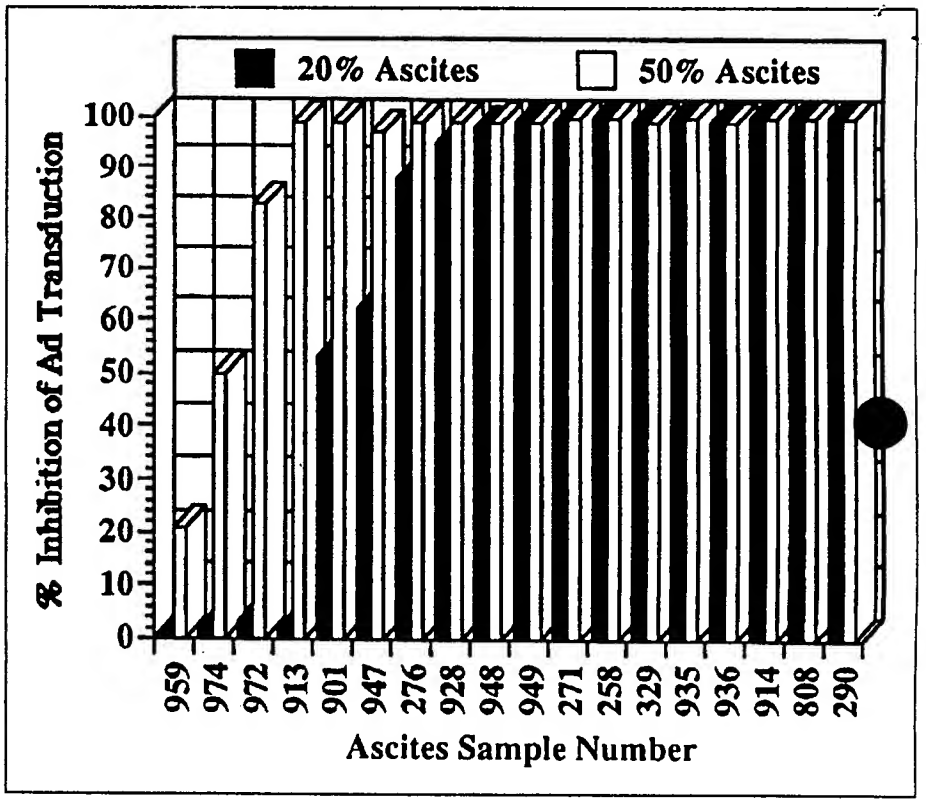
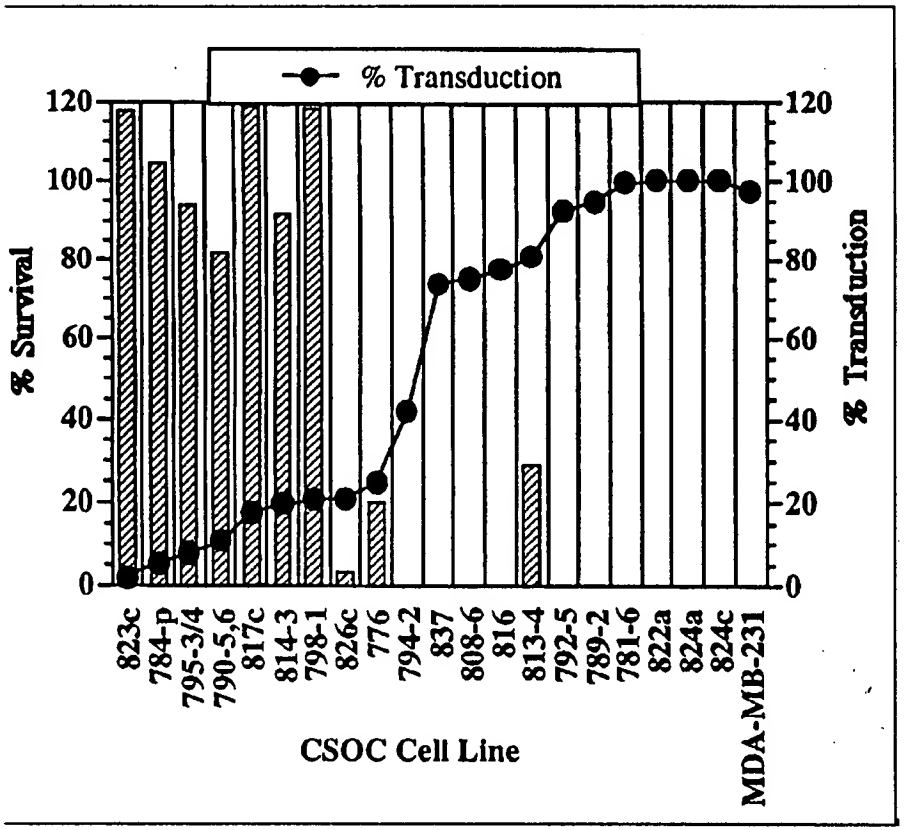


Fig. 32.1. Effect of malignant ascites on Adβ-Gal transduction of p53^{mut} MDA-MB-231 breast carcinoma cells. Patient biopsies had ovarian papillary serous histology. MDA-MB 231 cells were plated at 5 x 10^4 cells/well and allowed to adhere overnight. Next, 20% or 50% ascites in culture medium and 1 x 10^9 viral particles Adβ-Gal were added to each well for a final volume of 1ml. Twenty-four hours later, cells were fixed, assayed for β-galactosidase ac[redacted], and quantitated. The results shown are the means from triplicate wells. The % inhibition of Ad transduction was determined relative to MDA-MB-231 cells not exposed to ascites. These cells typically have 95-100% Ad transduction efficiency under these experimental conditions.

2.5 x 10^{12} pn, and 7.5 x 10^{12} pn given in single i.p. injections. The study goals of an acceptable safety profile and transgene expression in biopsy tissues were achieved despite the presence of anti-Ad antibodies prior to treatment. All patients had elevations in anti-Ad antibody levels subsequent to Ad p53 administration. The protocol design was then modified to allow for the addition of chemotherapy and multiple doses of Ad p53. The goals of the modification were to expand the safety profile of Ad-p53 to include multiple doses and to establish the safety profile of Ad p53 in combination with chemotherapy. The initial Ad p53 dosing level was 7.5 x 10^{12} pn daily (x2) in combination with i.p. cisplatin. An additional three patients received 2.5 x 10^{13} pn daily (x3) with i.p. cisplatin. An acceptable safety profile and transgene expression were both confirmed. Another modification was implemented in order to test intravenous carboplatin/paclitaxel instead of i.p. cisplatin while continuing to escalate the



ig. 32.2. Transduction of Cedars Sinai primary ovarian carcinoma cultures (CSOC) by dβ-Gal and tumor cell response to Ad-p53 treatment. Cells were cultured with 1 x 10^9 viral articles/ml Adβ-Gal or Ad-p53 for three days, then assayed for β-galactosidase activity or ell proliferation. Results are relative to untreated control. The plotted values are means from iplicate wells. (The standard errors were typically 2-5%).

ose of Ad-p53. Three patients received Ad p53 at 2.5 x 10^{13} pn with i.v. carboplatin (AUC f 6) and paclitaxel (175 mg/m^2). Then fifteen patients were dosed at 7.5 x 10^{13} pn daily (x5) lus i.v. carboplatin/paclitaxel. Accrual to this last dose level continues, but the preliminary ata indicates this regimen is well tolerated. Table 32.1 summarizes the dose escalation hema. Related serious adverse events included transient fever, increased liver function sts in one patient, abdominal distention, leukopenia, anemia, diarrhea, hypokalemia, ehydration, nausea and vomiting. All of these events were manageable, and other than one isode of elevation in alkaline phosphatase, did not preclude escalation to the next dose vel. One patient, who received Ad-p53 and chemotherapy, experienced a cascade of events ncluding sepsis, neutropenia, renal insufficiency, anemia, hypokalemia , nausea, vomiting, hrombocytopenia and edema. No evidence of viral shedding was seen in stool or urine. lthough this study was designed to address safety and biological activity, subjective evidence r tumor response was also noted. Preliminarily, there have been three subjective reductions

Table 32.1. Preliminary results from a Phase I gene therapy clincial trial for ovarian cancer

Patient No.	No. Doses	Dose (Viral Particles)	Chemotherapy	Transgene Expression
1	1	7.5 x 10^{10}	None	+
2	1	7.5 x 10^{10}	None	-
3	1	7.5 x 10^{10}	None	- (x2)
13	1	7.5 x 10^{10}	None	-
4	1	7.5 x 10^{11}	None	+
5	1	7.5 x 10^{11}	None	+
6	1	7.5 x 10^{11}	None	+
7	1	7.5 x 10^{11}	None	NR
8	1	7.5 x 10^{11}	None	+ (x1)
9	1	7.5 x 10^{11}	None	NR
10	1	2.5 x 10^{12}	None	+ (x1)
11	1	2.5 x 10^{12}	None	+ (x1)/- (x2)
12	1	2.5 x 10^{12}	None	+ (x1
14	1	7.5 x 10^{12}	None	-
15	1	7.5 x 10^{12}	None	+
16	1	7.5 x 10^{12}	None	-
207	2	7.5 x 10^{12}	IP cisplatin	BQL (C1)
212	2	7.5 x 10^{12}	Ip cisplatin	NR
17	3	2.5 x 10^{13}	Ip cisplatin	ND (C1), + (C2 and 3)
18	3	2.5 x 10^{13}	IV Carbo/ Taxol	Pending
19	3	2.5 x 10^{13}	IV Carbo/ Taxol	+ (C1 and 2)

ıble 32.1. Preliminary results from a Phase 1 p53 gene therapy clinical trial for ovarian cancer; cont.

'atient No.	No. Doses	Dose (Viral) Particles)	Chemotherapy	Transgene Expression
216	3	2.5×10^{13}	IV Carbo/Taxol	+ (C1)
20	3	2.5×10^{13}	IV Cisplatin/ Taxol	- (C1),+(C2)
21	3	2.5×10^{13}	IV Carbo/ Taxol	+ (C1 and 2)
22	3	2.5×10^{13}	IV Carbo/ Taxol	+/- (C1)*
23	5	7.5×10^{13}	IV Carbo/Taxol	Pending

R= sample degraded, no results; BQL= below quantifiable levels; ND= not done; C=dosing /cle; Patient 20 was allergic to carboplatin. *Ascites positive for transgene expression, tumor iopsy negative

ı ascites, one decreased CA-125, and one short lived CT-objective decrease in tumor mass. uture development of this treatment modality remains promising.

Conclusion

Seldom does such a new and exciting therapeutic category of drug make it into the linic. We are just starting to evaluate the extent to which p53 gene therapy can achieve linically meaningful outcomes and add to our currently inadequate cancer treatments. Key ɔ this discussion is the definition of appropriate clinical endpoints for gene therapy trials.)evelopment in an unprecendented area results in reliance on endpoints used in the past to ıstify approval of more traditional forms of cancer therapy. Historically, improvement in verall or disease-free survival has been the clinical "gold standard". Most practitioners would cknowledge that there are other meaningful endpoints which guide them in the care of ıeir patients. Endpoints such as improvement in quality of life and response rate may ranslate into an improvement in signs and symptoms of the disease. Other surrogates, such s improvement in tumor markers like CA-125, which parallels tumor burden, are significant ɔ the patient yet more difficult to prove to regulatory authorities. In the gene therapy arena, ther potentially meaningful endpoints include the ability to express the transgene, and lownstream effects such as tumor cell apoptosis. It must still be determined whether clinically ıeaningful results and regulatory requirements could include the combination of a surrogate ıarker, such as CA-125, and cellular apoptosis. Survival studies are extremely long and ignificantly delay the introduction of new therapeutics to the patient population. There is mple evidence that the use of replacement gene therapy, either alone, or in combination /ith chemotherapy, translates into anticancer effects. The preliminary clinical results of ransgene expression hold out hope that future applications in the clinic will result in mprovement in the current response and survival rates for cancer patients.

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Abbreviations

$p53^{null}$	no p53 protein expressed
$p53^{mut}$	mutant p53 protein expressed
$p53^{wt}$	wild type p53 expressed, but not necessarily functional; ciu, cellula infectious units
moi	multiplicity of infection = ciu/cell
pn	viral particles.
AUC	mg/ml/min = "area under the curve" of the carboplatin serun concentration versus time plot. Because elimination o fcarbo in i almost entirely dependent on renal glomerular filtration rate (in clearance rate in ml/min), carboplatin dosing is basedon the projecte area under the curve of the pharmacokinetic plot for each individua patient based on direct measurement of their creatine clearance or a indirect estimate of renal function based on patient age, mass, an serum creatinine concentration.

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Exhibit C

C

RAPID PUBLICATION

Phase II Trial of Intratumoral Administration of ONYX-015, a Replication-Selective Adenovirus, in Patients With Refractory Head and Neck Cancer

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Purpose: **To determine the safety, humoral immune response replication, and activity of multiple intratumoral injections of ONYX-015 (replication selective adenovirus) in patients with recurrent squamous cell carcinoma of the head and neck (SCCHN).**

Patients and Methods: **This phase II trial enrolled patients with SCCHN who had recurrence/relapse after prior conventional treatment. Patients received ONYX-015 at a dose of 2×10^{11} particles via intratumoral injection for either 5 consecutive days (standard) or twice daily for 2 consecutive weeks (hyperfractionated) during a 21-day cycle. Patients were monitored for tumor response, toxicity, and antibody formation.**

Results: **Forty patients (30 standard and 10 hyperfractionated) received 533 injections of ONYX-015. Standard treatment resulted in 14% partial to complete regression, 41% stable disease, and 45% progressive disease rates. Hyperfractionated treatment resulted in 10% complete response, 62% stable disease, and 29% progressive disease rates. Treatment-related toxicity included mild to moderate fever (67% overall) and injection site pain (47% on the standard regimen, 80% on the hyperfractionated regimen). Detectable circulating ONYX-015 genome suggestive of intratumoral replication was identified in 41% of tested patients on days 5 and 6 of cycle 1; 9% of patients had evidence of viral replication 10 days after injection during cycle 1, and no patients had evidence of replication ≥ 22 days after injection.**

Conclusion: **ONYX-015 can be safely administered via intratumoral injection to patients with recurrent/refractory SCCHN. ONYX-015 viremia is transient. Evidence of modest antitumoral activity is suggested.**

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THE PROGNOSIS FOR recurrent squamous cell carcinoma of the head and neck (SCCHN) region is discouraging.[1,2] Local tumor progression leads to morbidity and even death in the majority of patients. Therefore, improved local and local-regional therapeutic approaches are needed. Treatment after failure of surgery and radiation therapy generally involves chemotherapy.[1,2] Approximately 30% to 40% of patients with recurrent head and neck cancer respond to combination chemotherapy, which generally includes cisplatin. The duration of response is short, and median survival is less than 6 months.[1-9] Furthermore, local expansion of disease during or after chemotherapy leads to devastating functional, economic, cosmetic, and psychologic effects to the patient. Because recurrence frequently occurs within a prior radiation field, further radiotherapy is not an option, and palliative surgery is generally associated with excess morbidity and additional cost, while not affecting survival. Second-line chemotherapy with other agents, such as paclitaxel, docetaxel, methotrexate, topotecan, or gemcitabine (alone or in combination), has been tested, but response rates remain poor,[10-31] and the duration of the response is less than a few months.[9,32,33] Therefore, novel approaches to the local control of chemotherapy resistant/refractory SCCHN are needed.

ONYX-015 (dl1520) is a replication-selective adenovirus.[34] Efficient adenovirus replication is dependent on the expression of proteins that inactivate *p53*.[35,36] The normal *p53* gene product inhibits viral replication. ONYX-015 is an adenovirus that has been modified by deletion of the E1B 55-kd DNA fragment. The E1B–55-kd gene product inactivates *p53* in complex with E4ORF6.[37] It has been hypothesized that deletion of the E1B–55-kd region enables the p53 protein to maintain its function, thereby inhibiting viral replication in cells with normal *p53* function; however, in cells that lack normal *p53* function, such as malignant cells, the E1B–55-kd gene product may be expendable and the cells should be susceptible to replication and killing after infection.

Initial reports[38] suggested that *p53* mutant tumor cells could be lysed in a replication-dependent fashion both in

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vitro and in vivo after exposure to ONYX-015.[34,39] In addition, several tumor lines containing a normal wild-type *p53* gene sequence were also found to be sensitive to the oncolytic activity of ONYX-015.[39-41] This finding is expected, since *p53* function can be lost through multiple mechanisms besides gene mutation (eg, p53 protein binding degradation). Importantly, most groups found significantly less replicative capacity of ONYX-015 in weak normal cells compared with malignant cells,[39,41,42] which suggests a possible therapeutic index to ONYX-015 in the treatment of cancer.

Phase I investigation identified the toxicity of intratumoral injection of ONYX-015 to be limited to transient low-grade fever and injection site pain in one third of patients (S. Kaye, manuscript in preparation). Viral doses up to 1×10^{11} plaque-forming units (pfu) given daily once every 3 weeks, or 1×10^{10} pfu for 5 consecutive days every 3 weeks, were well-tolerated. No dose-limiting toxicity or maximum-tolerated dose was identified. Dose escalation, therefore, proceeded to the highest dose that could be practically manufactured. Additionally, multiday dosing with each dose administered to separate tumor quadrants seemed to be associated with a more effective induction of tumor necrosis over single-day dosing. Thus, we initiated a phase II investigation with ONYX-015 to be administered by intratumoral injection with multiple doses per cycles to patients with recurrent or refractory SCCHN.

PATIENTS AND METHODS

Enrollment Criteria

Patients were required to have histologically confirmed SCCHN (excluding nasopharyngeal) that had (1) recurred/relapsed after surgery and/or radiotherapy for the primary tumor and (2) had progressed on or within 8 weeks after completion of chemotherapy and/or radiotherapy (ie, tumors were refractory). Tumors could not be surgically curable. The tumor mass to be treated with ONYX-015 had to be adequately injectable (as defined below) and measurable (radiographically or by physical examination). Patients had to be older than 18 years old and had to have a Karnofsky performance status score of $\geq$ 70 and life expectancy of $\geq$ 3 months. Normal hematologic function and renal function were also required. A signed consent form (internal review board–approved) was required before enrollment. The *p53* gene status was not used as an enrollment criterion. Institutional review board approval of the protocol and consent form was required.

Baseline Assessment

Baseline assessments were made before treatment. Baseline *p53* gene sequencing and immunohistochemistry were performed on paraffin-embedded or frozen (−70°C) tumor material used for diagnosis of recurrence (when available). Baseline blood tests were performed that included complete blood counts, CD3, CD4, and CD8 lymphocyte counts, electrolytes, blood urea nitrogen, creatinine, and liver function tests. In addition, baseline neutralizing antibody titers to ONYX-015 were determined (most adults have neutralizing antibodies to the adenovirus type 5 coat proteins that are present on ONYX-015). In addition, flow cytometry was performed to determine circulating levels of CD3, CD4, and CD8 cells at baseline.

Fig 1. Schematic of the ONYX-015 detection amplicon. Nucleotides 2,453 to 2,544 of ONYX-015 are shown (5' strand only). This is the amplicon amplified in the TaqMan assay. The capital letters represent the sequence of the *Pub*-derived insert in construction of this virus. The underlined regions correspond to the 5' primer, the TaqMan probe, and the 3' primer. The probe and the 3' primer are homologous to the 3' strand.

ONYX-015

ONYX-015 (dl1520, also known as Cl-1042) is a chimeric human group C adenovirus (Ad2 and Ad5) that does not express the 55-kd product of the *E1B* gene (Pfizer, Inc, Ann Arbor, MI, and Onyx Pharmaceuticals, Richmond, CA).[37] It contains a deletion between nucleotides 2,496 and 3,323 in the *E1B* region encoding the 55-kd protein. In addition, a C-to-T transition at position 2,022 in *E1B* generates a stop codon at the third codon position of the protein. These alterations eliminate expression of the *E1B*–55-kd gene in ONYX-015–infected cells. ONYX-015 was grown and titered on the human embryonic kidney cell line HEK293.

Detection of ONYX-015 Adenovirus

The TaqMan assay is designed to amplify an amplicon of 92 nts (nts 2,453 to 2,544) that is specific for ONYX-015 (Fig 1). The specific detection of ONYX-015 is due to two factors: the amplicon overlaps the *E1B* region deletion (911 nts are missing from the wild-type sequence) and an 8-base pair *Puc*-derived linker insert is part of the TaqMan probe. The lower limit of quantitation for the assay is 4.2×10^4 particles of ONYX-015 per mL of plasma. The lower limit of detection is 1.05×10^4 particles of ONYX-015 per mL of plasma. This assay is specific for ONYX-015 DNA and does not detect wild-type adenovirus sequences. Polymerase chain reaction (PCR) cycling conditions are as follows: hold at −50°C, 2 minutes; hold at −95°C, 10 minutes; 40 cycles at −95°C, 15 seconds; and −63°C. The presence of PCR inhibitors in the sample is monitored using an independent PCR reaction.

Patient samples are spiked with exogenous DNA to monitor recovery in the extraction step and the presence of PCR inhibitors. A standard curve is prepared by serial diluting ONYX-015 virus from 2×10^9 to 1.05×10^4 vp/mL. Negative controls consist of a plasma control without virus and a type D adenovirus wild-type control. Viral DNA is extracted from patient samples, standard, and controls using a QIAamp DNA mini kit (Valencia, CA). The amount of ONYX-015 viral DNA is then quantitated by reverse transcription PCR using the above-described specific primer and probe.

ONYX-015 Handling and Processing

ONYX-015 is formulated as a sterile viral solution in Tris buffer (Tris 10 mmol/L [pH 7.4], $MgCl_2$ 1 mmol/L, CaCl 150 mmol/L, and 10% glycerol). The solution is supplied frozen (−20°C) in single-use, plastic screw-cap vials. Each vial contains 0.5 mL of virus solution at a specified viral titer. Vialed virus solution was thawed and diluted to the appropriate titer for dosing and was then further diluted to a final volume equivalent to 30% of the volume of the tumor to be injected. All dilutions were made with D5W (Baxter D5W electrolyte no. 45). Tumor volume was estimated by taking the product of the maximal

tumor diameter, its perpendicular and estimated depth, and dividing by 2. Vials of ONYX-015 were opened and diluted immediately before injection in biologic safety cabinets at the patient treatment area. All waste items were disposed in biohazard containers and autoclaved or incinerated.

Treatment Regimen

To ensure uniform dosing to the injected tumor in each patient, a single tumor was identified for ONYX-015 injection in each patient. If more than one injectable tumor was present, the most symptomatic and/or largest tumor mass was injected with ONYX-015. The tumor was mapped into five equally spaced and equally sized sections. Local anesthesia was applied to the skin as needed. The tumor was injected once a day (standard schedule) or twice a day (hyperfractionated schedule) with 10^{10} particles into each of the five quadrants. The suspension volume of D5W saline used for ONYX-015 administration was normalized to 30% of the estimated volume of the tumor mass to be injected (see above). During each treatment session, one puncture of the skin was made at a site approximately 80% of the distance from the tumor center out to the tumor periphery. Six to eight needle tracts were made radially out from the puncture site; virus was administered equally along the length of the needle tracts (25-gauge needle). This approach was carried out each day from puncture sites that were equally spaced out and encompassed the entire tumor mass. The majority of the viral dose was administered at the tumor periphery and at the interface between normal tissue and tumor tissue.

In the initial phase of the study, tumor injections were performed once daily for 5 consecutive days (standard schedule); these injections were repeated every 3 weeks or until tumor progression. After documentation of safety with this regimen (n = 30), a more aggressive injection regimen was tested in a subsequent 10 patients by administering a four-fold higher dose; identical injections were performed twice daily for 5 days during each of the first 2 weeks on study (hyperfractionated schedule). After a 1-week rest period, the hyperfractionated regimen was repeated. Patients' vital signs were taken 15 minutes before and after each treatment for a minimum of 30 minutes. Patients were eligible for repeat treatment cycles at the same dosage every 3 weeks if no grade 4 toxicity with the prior treatment cycle of ONYX-015 occurred and no progression of the injected tumor was observed. After this induction regimen, maintenance treatment cycles were given by the same schedule as was used in the initial patient cohort (every 3 weeks as described above). The injections were given in outpatient clinics, including Mary Crowley Medical Research Center at Baylor University Medical Center (Dallas, TX), Albany Regional Cancer Center (Albany, NY), US Oncology Research (Houston, TX), Beatson Oncology Centre (Glasgow, Scotland), Dana-Farber Cancer Institute (Boston, MA), University of Chicago (Chicago, IL), and M.D. Anderson Cancer Center (Houston, TX).

Tumor Assessments

Tumor masses were measured serially by either physical examination or radiographic scanning (computed tomography or magnetic resonance imaging), whichever the principal investigator deemed most accurate for the measurement of the injected tumor mass. In general, very superficial lesions were measured by physical examination and deeper tumors were measured most accurately by radiographic scanning. Tumor measurements were determined either every 3 weeks (physical examination) or every 6 weeks (computed tomography/magnetic resonance imaging scans) while patients were on active study treatment; after treatment completion, patients' tumors were assessed every 8 weeks or sooner if signs or symptoms of progression became evident. Radiographic scans were assessed by independent radiologists who were not investigators on the study.

The degree of necrosis induction within injected tumors was categorized as follows: complete regression, complete disappearance of measurable tumor; partial regression, ≥ 50% but less than 100% decrease in nonnecrotic cross-sectional tumor area; minor response, less than 50%, ut ≥ 25% decrease in nonnecrotic tumor area; stable disease, less than 25% decrease and less than 25% increase in nonnecrotic tumor area; progressive disease, ≥ 25% increase in tumor area versus the baseline area. Radiologists were blinded to the *p53* gene status and neutralizing antibody titer of the patients at the time of tumor assessment. Tumors were considered assessable for response at earliest assessment at any time after the first injection. All lesions (injected/noninjected) were followed to assess response.

p53 *Gene Sequencing Determination*

Exons 5 through 9 of the tumor *p53* gene were sequenced completely during the first two thirds of the trial. Exons 2 through 11 were assessed by *p53* gene chip technology during the final one third of the trial. Since certain gene deletions can be missed by gene chip analysis (ie, a wild-type sequence is reported despite a functionally significant mutation), wild-type *p53* gene sequences by gene chip analysis underwent confirmatory sequencing to be validated.

Determination of Neutralizing Antibody Titers

Patient and control samples were incubated at 55°C for 30 minutes to inactivate complement. Clinical plasma samples previously determined to produce high, mid-range, and negative titers were designated as plasma controls. Each dilution was mixed with adenovirus stock at a titer prequalified to produce 15 to 20 plaques per well of a 12-well dish in DMEM growth medium. The patient samples and controls were inoculated for 1 hour at room temperature and applied to 70% to 80% confluent JH293 cells in 12-well dishes. After 2 hours of incubation at 37°C, 5% CO_2 plasma-virus mix was removed and 2 mL of 1.5% agarose in DMEM was added to each well. Plates were read on day 7 after inoculation by counting the number of plaques per well. The titer of neutralizing antibody for each sample was reported as the dilution of plasma that reduced the number of plaques to 60% of the number of plaques in the virus control without antibody. Determinations of neutralizing antibody titers were made before cycle 1 (baseline), before cycle 2, and before cycle 3.

Additional Follow-Up After Treatment Initiation

Neutralizing antibody titers were repeated every 4 weeks. Routine blood testing, including complete blood count and differential, electrolytes, blood urea nitrogen, creatinine, and liver function tests, was repeated every 3 weeks. Blood samples to determine circulating ONYX-015 after intratumoral injection at cycle 1 were determined on days 1 and 5.

RESULTS

Treatment

Forty patients were entered onto the trial from six sites (Mary Crowley Medical Research Center at Baylor University, M.D. Anderson Cancer Center, Beatson Cancer Institute, Albany Regional Cancer Center, Dana-Farber Cancer

Table 1. Patient Demographics

	ONYX-015 Standard Schedule (qd × 5/21-day cycle)		ONYX-015 Hyperfractionated Schedule (bid × 10/21-day cycle)	
	No. of Patients	%	No. of Patients	%
No. of patients registered	30		10	
Age, years				
Median	58		70	
Range	39-74		49-78	
Sex				
Male	23	77	8	80
Female	7	23	2	20
KPS score				
90-100	16	54	2	20
70-80	14	46	8	80
Prior therapy				
Surgery	21	70	7	70
Radiotherapy	28	93	10	100
Chemotherapy	19	63	6	60
≥ 2 modalities	27	90	8	80
Location of recurrence*				
Larynx	4	13	2	20
Tongue	2	7	0	0
Cervical area	17	57	1	10
Other	7	23	7	70
Tumor size, cm				
Maximum area				
Median	12.76		10.99	
Range	1.56-38.50		1.1-69	
Maximum diameter				
Median	3.4		3.85	
Range	1-8.4		0.9-7	
Baseline neutralizing < 1:20	14	47	2	22
Antibody levels ≥ 1:20	16	53	7	78
Baseline CD4 counts				
Median	334.22		294	
Range	79.24-1,318.05		188-798	
p53 gene status				
Mutant	12	40	6	60
Wild-type	11	37	2	20
Undetermined	7	23	2	20

Abbreviation: KPS, Karnofsky performance status.

*Two patients had distant pulmonary metastases that were not injected.

Institute, the University of Chicago) between July 1997 and September 1998. The first 30 patients were enrolled onto the standard ONYX-015 schedule trial; the 10 patients enrolled subsequently received the hyperfractionated regimen. All patients registered received at least a single injection of ONYX-015 and were assessable for toxicity. Thirty-six patients were considered assessable for initial response. Two patients (one standard, one hyperfractionated) were not assessable due to death before response assessment (not treatment-related), and two patients (hyperfractionated) withdrew before response assessment. Characteristics of patients receiving the standard versus hyperfractionated schedule are listed in Table 1. As listed in Table 2, 70 cycles (345 doses) were administered to 30 patients who received standard-schedule ONYX-015, and 27 cycles (188 doses) were administered to 10 patients who received the hyperfractionated schedule.

Tumor Response

Data on the response of injected tumors is listed in Table 3. Four patients (14%) who received the standard dosing schedule achieved a partial or complete regression of the injected tumor, 12 (41%) had stable disease, and 13 (45%) progressed. One (14%) of the hyperfractionated patients achieved a complete regression, four (58%) achieved stable disease, and two (29%) progressed. The median time to

Table 2. Treatment Parameters

	ONYX-015 Standard Schedule	ONYX-015 Hyperfractionated Schedule
No. of cycles per patient		
Median	2	2
Range	1-8	1-6
Total number of cycles	70	27
No. of injections	325	188
No. of days on study per patient		
Median	35.5	87.5
Range	13-175	40-254
Dilution volume, ml per patient per injection		
Median	6.05	5.85
Range	0.1-53.1	0.1-42.4

injected tumor progression, progression-free survival, and survival with the standard versus hyperfractionated approaches are listed in Table 3. No significant differences were observed between the two dosing regimens. There was no correlation between baseline tumor area, neutralizing antibody level, and response. A significant correlation was demonstrated between antitumoral activity (complete, partial, and minor responses) and presence of a *p53* gene mutation ($P = .017$).

Toxicity

Toxicity that occurred in more than 25% of patients is listed in Table 4. The majority of the toxic effects were of mild or moderate intensity. Fatal toxicity not related to ONYX-015 occurred in three standard-treatment patients (10%) and one hyperfractionation-treated patient (10%). One fatality was related to hematemesis from an unrelated gastrointestinal ulcer, one was due to hemorrhage from local progression, one was due to bacterial-induced septic shock, and one was due to anoxia caused by airway obstruction from progressive disease. Fourteen serious adverse events were reported in the standard arm, and nine serious adverse events occurred in the hyperfractionated arm. One serious adverse event was categorized as "probably related" to study medication in the standard arm (hemorrhage at injection site). The following events were categorized as possibly related to ONYX-015 injection: pneumonia with no organisms identified (n = 1), confusion (although concurrent hypocalcemia may have been related; n = 1), and recurrent atrial flutter (n = 1). The other 10 serious adverse events were either not related or the relationships were unable to be determined. Among patients who received hyperfractionated treatment, one patient developed injection site hemorrhage categorized as possibly related to ONYX-015 injection. Other events were categorized as not related or unable to be determined. Nine of 40 patients developed pneumonia not related to study treatment (six standard and three hyperfractionated). A specific cause of pneumonia was identified in three patients (two bacterial and one unrelated peptic ulcer perforation). The six other causes were thought to be related to aspiration associated with the cancer. The pneumonia lasted from 6 to 13 days. Six patients were retreated with ONYX-015 after resolution of pneumonia without recurrence. Reasons for study discontinuation are listed in Table 5.

Table 3. Response of Injected Lesions

	ONYX-015 Standard	ONYX-015 Hyperfractionated
Complete response	2 (7)	1*
Partial response	2 (7)	0
Minor response	2 (7)	1
Stable disease	10 (34)	3
Progressive disease	13 (45)	2
Not assessable	1†	3†,‡
Median progression-free survival§, days:		
Responders (PR, CR, MR, SD)	59	80
Nonresponders	21	16.5
Nonassessable	—	5
All	22	53
Median survival§, days:		
Responders (PR, CR)	125	152
Nonresponders	183	58
Nonassessable	25	—
All	126	75

Abbreviations: PR, partial response; CR, complete response; MR, minor response; SD, stable disease.

*Complete histologic response at autopsy.

†Death not related to treatment occurred before tumor assessment.

‡Patient withdrew before response assessment.

§Derived by the Kaplan-Meier product-limit method.

Systemic Distribution of ONYX-015

All 30 patients (29 of 30 in cycle 1) in the standard arm were tested for circulating ONYX-015 using PCR analysis 24 hours after the 5-day intratumoral injection series. Previous studies have shown rapid clearance of the ONYX-015 genome from the blood (approximately 6 hours); therefore, viremia ≥ 24 hours after the last injection is strong evidence for viral replication and shedding. Detectable levels of ONYX-015 were identified in 12 (41%) of 29 patients 24 hours after the last ONYX-015 injection (Table 6). In two patients (9%), the ONYX-015 genome was detected 10 days after injection in cycle 1. No samples were positive for circulating ONYX-015 genome 22 days after any injection in cycle 1 or any other cycle, and 15 days after any injection beyond cycle 1. Six (28%) of 21 patients had detectable circulating ONYX-015 genome in cycle 2 24 hours after intratumoral injection, and two of eight patients had detectable circulating ONYX-015 genome 24 hours

Table 4. Toxicity Occurring in More Than 25% of Patients Possibly Related to Study Treatment

Toxicity	Grade						Total	
	Mild	Moderate	Severe	Life Threatening	Fatal*	Unknown	No.	%
Standard approach (n = 30)								
Fever	9	12	0	0	0	1	22	73
Asthenia	5	5	5	0	0	0	15	50
Chills	7	6	2	0	0	0	15	50
Injection site pain	1	7	6	0	0	0	14	47
Headache	1	5	3	0	0	0	9	30
Nausea	1	4	4	0	0	0	9	30
Dyspnea	0	6	2	0	0	0	8	27
Hyperfractionated approach (n = 10)								
Fever	1	4	1	0	0	0	6	60
Injection site pain	6	2	0	0	0	0	8	80
Asthenia	1	4	1	0	0	0	6	60
Headache	1	2	0	0	0	0	3	30
Bacterial infection	1	2	0	0	0	0	3	30
Depression	2	1	0	0	0	0	3	30
Respiratory disorder	1	0	2	1	0	0	4	40
Pneumonia	0	2	1	0	0	0	3	30
Nausea	3	3	1	0	0	0	7	70
Anorexia	3	0	0	0	0	0	3	30
Hypotension	0	1	1	2	0	0	4	40
Hemorrhage	1	0	1	1	0	0	3	30
Tachycandia	2	0	1	0	0	0	3	30
Hyponatremia	0	3	0	1	0	0	4	40
Sweating	2	1	0	0	0	0	3	30
Anemia	0	1	2	0	0	0	3	30

*In the standard approach group, death that occurred in three patients was related to hematemasis, hemorrhage, and bacterial sepsis and was not related to study medication. Among the patients treated with the hyperfractionated schedule, death occurred in one patient due to bronchial obstruction induced by progressive disease.

after injection in cycle 3. The two patients who had detectable ONYX-015 genome in cycle 3 achieved a minor response and a complete response. Otherwise no correlation between circulating genome and response was observed in patients with detectable genome in cycle 2 or patients with circulating genome in cycle 1. Patients entered onto the hyperfractionated treatment arm were not followed for systemic distribution of ONYX-015 genome.

Table 5. Reasons for Study Discontinuation

	Standard		Hyperfractionated	
	No. of Patients	%	No. of Patients	%
Disease progression at injected site	10	33	1	10
Disease progression at noninjected site	10	33	4	40
Patient decision	4	13	1	10
Unrelated medical condition(s)	1	3	0	0
Investigator's decision	1	3	2	20
Death	3	10	1	10
Other	1	3	1	10

Neutralizing Antibody Titers

Sixteen patients who received standard ONYX-015 and seven patients who received hyperfractionated ONYX-015 were identified as having high (elevated > 1:20) neutralizing antibody titers at baseline (Table 1). Fifty-three percent

Table 6. PCR-Detectable ONYX-015 Genome in Circulation After Intratumoral Injection

Cycle	Days 6 and 7		Day 15		Day 22	
	+	−	+	−	+	−
+	12	17	2	19	0	22
2	6	15	0	17	0	18
3	2	6	0	7	0	6
4	0	2	0	2	0	2
5	0	1	0	1	0	0
6	0	1	0	0	0	0
7	1	0	0	1	0	1
8	0	1	0	1	0	1

Symbols: +, number of patients with positive detectable genome; −, number of patients with genome not detectable.

of standard-arm patients had antibody titers more than 1:20 at baseline, and 23 (96%) of 24 patients measured after cycle 1 had antibody titers above 1:20. All patients in the standard arm had neutralizing antibody titers above 1:20 after cycle 2. The median antibody titers at baseline (n = 30) was 51 (range, 0 to 1,798). After cycle 1 (n = 24), the median titer was 11,896 (range, 0 to 81,920). After cycle 2 (n = 14), the median titer was 12,363 (range, 225 to 71,425). Similar titers were seen in the hyperfractionation-treated patients. At baseline (n = 9), the median neutralizing titer was 1,074 (range, 0 to 8,847). This increased to 9,733 (range, 2,165 to 62,700) after cycle 2 (n = 5). There was no correlation of baseline titer levels to tumor response, time to local progression, progression-free survival duration, or overall survival.

DISCUSSION

The results from these trials indicate that intratumoral injection of the replication-selective adenovirus ONYX-015 at a dose of 1×10^{10} pfu daily for 5 days of a 21-day cycle was well tolerated. Transient low-grade fever and injection site pain were the most frequent toxicities. These were manageable on an outpatient basis. Antitumor activity (as measured by $\geq$ 50% tumor destruction) was observed in approximately 14% of patients and did not seem different between the standard and hyperfractionation arms. Survival was also not different between the two arms; however, injection site pain occurred more frequently on the hyperfractionated regimen. Future proof of clinical benefit will be necessary to determine clinical utility. These data suggest that ONYX-015 has a favorable safety profile and modest efficacy in recurrent head and neck cancer as a single agent. Future testing in this patient population has, therefore, focused on combinations with standard agents, such as cisplatin-based chemotherapy.[43]

Replication-competent viruses have been tested as therapeutic agents for more than 100 years. Smallpox was eradicated with a replicating virus vaccine.[44,45] Exploration of the use of replicating viruses for the treatment of cancer was documented as early as 1912 when a woman with advanced cervical cancer achieved a response after injection with an attenuated rabies virus.[46,47] In 1950, the oncolytic activity of Egypt 101 virus was validated in vitro,[48] and clinical activity was suggested after intratumoral injection in cancer patients.[49-51] However, the antitumoral effects were transient (< 3 months). Subsequent clinical investigation with mumps virus as a cancer therapy was reportedly associated with a 41% "response" rate in 90 treated patients.[52] However, a follow-up trial[53] involving 200 cancer patients in whom mumps virus was administered by a multiple intratumoral injection schedule revealed transient tumor regression in only 26 patients. Toxicity was limited to transient fever and injection site pain. Another oncolytic virus, Newcastle disease virus (NDV),[54-58] showed selective replicative capacity in malignant cells. The mechanism of NDV selectivity may be related to elevated *myc* oncogene expression or differences in membrane permeability, as opposed to the *E1B*–55-kd deletion effect on *p53* with ONYX-015.[58-60] Additionally, consistent with what we observed with ONYX-015, tumor response was correlated with viral replication–induced oncolysis.[57] NDV was used to lyse tumor cells in vitro for the purpose of creating a viral oncolysate (virus and lysed tumor cells). Several trials in melanoma patients with limited-stage disease undergoing surgical resection followed by vaccination with the NDV viral oncolysate suggested improved survival compared with historical controls.[61-64] Similar results have been found in separate trials involving patients with colorectal carcinoma,[65] advanced renal cell carcinoma,[66] metastatic breast cancer, and ovarian cancer.[67] Influenza virus and vaccinia virus have also been studied as a viral oncolysate for tumor vaccine trials.[68-70] More recently, a variety of replication-selective viruses have been either engineered for replication selection (including human adenovirus, herpes virus, and vaccinia virus)[71] or shown to be replication-selective based on specific genetic tumor target (ie, activated *ras* for retrovirus).[72-75] Replication-selective, tumor-targeting bacteria such as *Salmonella typhimurium* have also shown encouraging preclinical activity.

A great deal of data have been accumulated suggesting that adenovirus serotype 5 is an effective oncolytic virus with a low toxicity profile to humans. DNA from thousands of human tumors have been analyzed for the presence of adenovirus DNA, and no integrated viral DNA has been isolated from any human tumor.[72] Eighty percent of adults have existing antibodies to adenovirus serotype 5, but less than 15% of exposed patients become clinically symptomatic.[73] The most common symptoms of an adenoviral serotype 5 infection are flu-like in nature and include cough, gastroenteritis, conjunctivitis, and, rarely, pneumonia. However, these symptoms are rarely seen even in immunocompromised patients.[74] Oral adenoviral vaccines were given to thousands of military recruits in the 1960s without adverse effects or increase in cancer.[75] Long- and short-term safety of intratumoral adenoviral injection has been shown in several animal cancer models,[76-82] and live adenovirus inocula were given intratumorally and intra-arterially to patients with cervical carcinoma at the National Cancer Institute in the 1950s.[51] Again, no significant toxicities, other than transient fever and malaise, were observed, even in subsets of patients treated with corticosteroids and in those in whom neutralizing adenovirus antibodies were not

present. Intravascular administration was also well tolerated in a small group of patients.[83] Adenoviral vectors with the E1 and E3 deletion containing the *Escherichia coli* cytosine deaminase gene have also been administered via intradermal injection to normal individuals in studies of toxicity and immune response at dose levels of 10^6, 10^7, and 10^8 pfu.[84] No significant toxicity was observed.[84] This was consistent with clinical trial results in the same patient population of head and neck cancer patients described in this trial who received a nonreplicating adenoviral vector containing a wild-type *p53* gene.[85,86]

Given the safety and toxicity profile of ONYX-015, it seems reasonable to explore this virus in patients with earlier-stage disease[87-90] and possibly even to enhance sensitivity when combined with chemotherapy or radiation therapy.[39] Independent of the ONYX-015 replication-induced oncolysis, ONYX-015 *E1A* gene expression can activate the cell cycle and increase cellular sensitivity to chemotherapy or radiation therapy.

Use of ONYX-015 for local management of SCCHN and as adjuvant therapy after surgical resection of SCCHN and, possibly, other malignant tumors should also be considered but will require further investigation. Comparison of survival between responding and nonresponding patients will also need to be followed in the future, although differences observed in this trial were not significant. Pursuit of other schedules of intratumoral administration (ie, > 5 days/21-day course) are unlikely to be of value, although justification of a systemic infusion schedule for ONYX-015 may be warranted since it has been shown to be safe and efficacious in animal cancer models.[39] Detection of ONYX-015 genome in plasma on the last day of ONYX-015 injection suggests that circulating virus, at low plasma concentration, is safe. Furthermore, persistent detection in two patients 10 days after the last injection suggests that a viral replicative process was ongoing, although it did not persist since none of the samples tested showed evidence of circulating viral genome more than 17 days after the last injection.

Future work with ONYX-015 and other replication-selective viruses will also explore the possibility of arming these viruses with exogenous genes, particularly if selective tumor replication is confirmed. Antitumor effects correlating with enhanced cytotoxic T-lymphocyte activity have been noted in vivo with replication-selective herpes simplex virus (G207) carrying an interleukin 12 gene,[71] for example. Over the next year, a number of these replication-selective agents are expected to enter clinical testing.

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Attachment

Response to Office Action
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Exhibit D

[CANCER RESEARCH 60, 6359–6366, November 15, 2000]

Selective Replication and Oncolysis in p53 Mutant Tumors with ONYX-015, an E1B-55kD Gene-deleted Adenovirus, in Patients with Advanced Head and Neck Cancer: A Phase II Trial

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ABSTRACT

ONYX-015 is an E1B-55kDa gene-deleted adenovirus engineered to selectively replicate in and lyse p53-deficient cancer cells. To evaluate the selectivity of ONYX-015 replication and cytopathic effects for the first time in humans, we carried out a Phase II clinical testing of intratumoral and peritumoral ONYX-015 injection in 37 patients with recurrent head and neck carcinoma. Patients received ONYX-015 at a daily dose of 1×10^{10} plaque-forming units (pfu) via intratumoral injection for 5 days during week 1 of each 3-week cycle ($n = 30$; cohort A), or 1×10^{10} pfu twice a day for 10 days during weeks 1 and 2 of each 3-week cycle. Posttreatment biopsies documented selective ONYX-015 presence and/or replication in the tumor tissue of 7 of 11 patients biopsied on days 5–14, but not in immediately adjacent normal tissue (0 of 11 patients; $P = 0.01$). Tissue destruction was also highly selective; significant tumor regression (>50%) occurred in 21% of evaluable patients, whereas no toxicity to injected normal peritumoral tissues was demonstrated. p53 mutant tumors were significantly more likely to undergo ONYX-015-induced necrosis (7 of 12) than were p53 wild-type tumors (0 of 7; $P = 0.017$). High neutralizing antibody titers did not prevent infection and/or replication within tumors. ONYX-015 is the first genetically engineered replication-competent virus to demonstrate selective intratumoral replication and necrosis in patients. This agent demonstrates the promise of replication-selective viruses as a novel therapeutic platform against cancer.

INTRODUCTION

The *p53* tumor suppressor gene is mutated in roughly 50% of all human cancers (1), including non-small cell lung (60%), colon (50%), breast (40%), head and neck (60%), and ovarian (60%) cancers in the advanced stages (2–6). Loss of p53 function is associated with resistance to chemotherapy and/or decreased survival in numerous tumor types, including breast (7), colon (8), bladder, ovarian (9), and non-small cell lung cancers (10). Therefore, effective therapies for tumors that lack functional p53 are clearly needed.

Many of the same critical regulatory proteins that are inactivated during carcinogenesis are also inactivated by adenoviral gene products during replication (10–13). Because of this convergence, the deletion of viral genes that inactivate these cellular regulatory proteins can be complemented by genetic inactivation of these proteins within cancer cells. An E1B-55kDa gene-deleted adenovirus, ONYX-015 (dl1520), is currently being developed for the treatment of tumors lacking p53 function (11, 14). The *p53* gene product is responsible for several growth-regulatory functions. One critical function includes induction of cell cycle arrest and/or apoptosis in response to foreign DNA synthesis (15–17). p53 can induce either a G_1 growth arrest by inducing cyclin-dependent kinase inhibitor p21/WAF1/Cip1 (18, 19), or apoptosis by inducing *bax*-1 (20) after a DNA virus infection. Because the E1B-55kD gene product is responsible for p53-binding and inactivation, it was hypothesized that an E1B-55kDa deletion mutant would be unable to inactivate p53 in normal cells and would, thus, be unable to replicate efficiently. In contrast, cancer cells lacking functional p53 (*e.g.*, because of gene mutation) would hypothetically be sensitive to viral replication and subsequent CPE.[2]

The original article describing this approach reported that p53 mutant tumor cells could be destroyed in a replication-dependent fashion both *in vitro* and *in vivo* (21). Many tumor cell lines with normal *p53* gene sequences, however, were subsequently found to be relatively sensitive to the effects of ONYX-015 *in vitro* (7 of 10 tested; Ref. 14), and subsequent publications from several other groups suggest that the *p53* gene sequence is a poor predictor of sensitivity to ONYX-015 *in vitro* (22–25). Given the numerous mechanisms by which *p53* can be inactivated besides genetic mutation, this finding is not necessarily inconsistent with the original hypothesis (6, 26). Factors which inhibit p53 protein function, despite the presence of a normal *p53* gene sequence, include expression of the human papillomavirus E6 protein or *mdm-2* gene amplification (27). Additionally, p14arf is known to be deleted in the vast majority of tumor cell lines with normal *p53* gene sequences (28, 29). Even normal cells that are placed into tissue culture can rapidly select for a loss of p16 and p14arf during immortalization. Because the adenoviral E1A gene product can cause p53 induction through p14arf, the loss of p14arf may also reduce the ability of p53 to block ONYX-015 replication in some p53 wild-type tumor cells.[3]

One approach to studying the mechanism of ONYX-015 selectivity was to compare its behavior in cell lines that are identical except for p53 function. Four matched pairs of cell lines have confirmed that ONYX-015 replication and/or CPEs are significantly inhibited by functional p53: RKO (21), H1299 (30), A2780,[4] and U343.[5] In contrast to these four cell lines, however, a fifth (U2OS) cell line did not become significantly more sensitive to ONYX-015 after transfection with dominant-negative p53 (31). Although it is possible that as-yet-undetermined complementing mutations within tumor cell lines may account for these differences in ONYX-015 effects, most data confirm the selective replication capacity of ONYX-015. Some of the controversy regarding ONYX-015 selectivity to p53 mutant targets may be related to the multiplicity of infection used, the study end points, and the time elapsed from infection to assessment of CPEs (32).Overall, selectivity of ONYX-015 replication appears to be most consistent at low multiplicities of infection ($\leq$1.0 pfu) after prolonged observation of CPE assays.

We carried out initial clinical testing of ONYX-015 in patients with recurrent squamous cell carcinoma of the head and neck. Abnormal-

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[2] The abbreviations used are: CPE, cytopathic effect; pfu, plaque-forming unit(s).

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[4] I. Ganly, personal communication.

[5] S. Freytag, personal communication.

ities in p53 function are common as a result of gene mutation or protein degradation attributable to human papillomavirus E6 protein expression (33, 34). Phase I investigation indicated good tolerability of ONYX-015 administered as a single intratumoral injection (doses up to 10^{11} pfu), and after a series of five daily consecutive intratumoral injections (doses up to 10^{10} pfu per injection (35). Injections were done and the tumors were biopsied in an outpatient setting, which allowed clinical and histological assessment over time after tumor inoculation. Local-regional tumor progression was the cause of morbidity and death in the majority of cases. We, therefore, could obtain valuable biological data on viral presence, necrosis, and inflammation while attempting to benefit patients through local tumor control in Phase II testing.

MATERIALS AND METHODS

Enrollment Criteria. All of the head and neck cancer patients participating in Phase II studies of intratumoral injection with ONYX-015 as a single agent were analyzed. Patients had histologically confirmed squamous cell carcinoma of the head and neck (excluding nasopharyngeal) that had recurred after surgery and/or radiotherapy for the primary tumor and had progressed at or within 8 weeks after completion of chemotherapy and/or radiotherapy (*i.e.*, tumors were refractory). Tumors could not be surgically curable. The tumor mass to be treated with ONYX-015 had to be adequately injectable (as defined below) and measurable (radiographically or by physical examination). Patients had to be ≥18 years old, had to have a Karnofsky performance status of ≥70%, and life expectancy of ≥3 months. Normal hematological and renal function were also required. This investigation was performed after approval by the local Institutional Review Board at US Oncology (Dallas, TX). An informed consent was obtained from each patient or from the patient's legal guardian prior to enrollment. The *p53* gene status was not used as an enrollment criteria. Institutional Review Board approval of the protocol and consent form was required.

Baseline Assessment. Baseline assessments were made prior to treatment, but these results were not used as enrollment criteria. A biopsy sample was obtained for *p53* gene sequencing from the tumor to be injected (see methods below). Baseline blood tests were performed as follows: complete blood counts; CD3, CD4, and CD8 lymphocyte counts; electrolytes; blood urea nitrogen; creatinine; and liver function tests. In addition, baseline neutralizing antibody titers to ONYX-015 were determined (most adults have neutralizing antibodies to the adenovirus type 5 coat proteins that are present on ONYX-015). In addition, delayed-type hypersensitivity skin testing (Merieoux) and plain chest radiographs were performed.

ONYX-015. ONYX-015 (dl1520) is a chimeric human group C adenovirus (Ad2 and Ad5) that does not express the product of the E1B-55kDa gene; the virus was constructed in the laboratory of Arnold Berk (Barker and Berk, Ref. 11). The virus contains a deletion between nucleotides 2496 and 3323 in the E1B-55kDa region encoding the protein. In addition, a C to T transition at position 2022 in E1B generates a stop codon at the third codon position of the protein. These alterations eliminate expression of the E1B-55kDa gene in ONYX-015 infected cells. ONYX-015 was grown and titered on the human embryonic kidney cell line HEK293 as described previously (14).

ONYX-015 Handling and Processing. ONYX-015 is formulated as a sterile viral solution in TRIS buffer [10 mM TRIS (pH 7.4), 1 mM $MgCl_2$, 150 mM CaCl, and 10% glycerol]. The solution is supplied frozen (−20°C) in single-use, plastic screw-cap vials. Each vial contains 0.5 ml of virus solution at a specified viral titer. Vialed virus solution was thawed and diluted to the appropriate titer for dosing, and was then further diluted to a final volume equivalent to 30% of the volume of the tumor to be injected. Tumor volume was estimated by taking the product of the maximal tumor diameter, its perpendicular and estimated depth, and dividing by two. Vials of ONYX-015 were opened and diluted immediately prior to injection in biological safety cabinets at the patient treatment area. All of the waste items were disposed of in biohazard containers and autoclaved or incinerated.

Treatment Regimen. To ensure uniform dosing to the injected tumor in each patient, a single tumor was identified for ONYX-015 injection in each patient. If more than one injectable tumor was present, the most symptomatic and/or largest tumor mass was injected with ONYX-015. The tumor was mapped into five equally spaced and equally sized sections. Local anesthesia was applied to the skin as needed. The tumor was injected with 10^{10} pfu following the template displayed in Fig. 1. The suspension volume of saline used for ONYX-015 administration was normalized to 30% of the estimated volume of the tumor mass to be injected (see above). During each treatment session, one puncture of the skin was made at a site approximately 80% of the distance from the tumor center out to the tumor periphery. Six to eight needle tracts were made radially out from the puncture site; virus was administered equally along the length of the needle tracks (25-gauge needle). This approach was carried out each day from puncture sites that were equiradially spaced out and that encompassed the entire tumor mass. The majority of the viral dose was administered at the tumor periphery and at the interface between normal tissue and tumor tissue. This administration approach was used for two reasons. First, prior studies have suggested improved efficacy with this administration approach. Second, this technique allowed for assessment of the effects of ONYX-015 injection on both normal tissues and tumor tissues in the same patients.

In the initial phase of the study, tumor injections were performed once daily for 5 consecutive days; these injections were repeated every 3 weeks or until tumor progression. After documentation of safety with this regimen, a more aggressive injection regimen was pilot-tested in subsequent patients; identical injections were performed twice daily for 5 days during each of the first 2 weeks on study. After a 1-week rest period, this regimen was repeated. Following this induction regimen, maintenance treatment cycles were given by the same schedule as was used in the initial patient cohort (every 3 weeks as described above). No significant complications related to injection were observed (36). The injections were given in the outpatient clinics including Beatson Cancer Institute (Glasgow, Scotland), M. D. Anderson Cancer Center (Houston, Texas), Mary Crowley Medical Research Center at Baylor University Medical center (Dallas, Texas), and Albany Regional Cancer Center (Albany, New York), which are investigative sites within US Oncology (Houston, Texas). Patients' vital signs were taken 15 min prior to and after each treatment for a minimum of 30 min. Patients were eligible for repeat treatment cycles at the same dosage every 3 weeks if no grade 4 toxicity (National Cancer Institute Common Toxicity Criteria) occurred with the prior treatment cycle of ONYX-015 and no progression of the injected tumor was observed.

Tumor Assessments. Tumor masses were measured serially by either physical examination or radiographic scanning (computed tomography or magnetic resonance imaging), whichever the principal investigator deemed most accurate for the measurement of the injected tumor mass. In general, very superficial lesions were measured by physical examination, and deeper tumors were measured most accurately by radiographic scanning. Tumor measurements were determined either every 3 weeks (physical examination) or every 6 weeks (computed tomography/magnetic resonance imaging scans) while patients were on active study treatment. After treatment completion, patient's tumor(s) were assessed every 8 weeks or sooner if signs/symptoms of progression became evident. Radiographic scans were assessed by independent radiologists who were not investigators on the study.

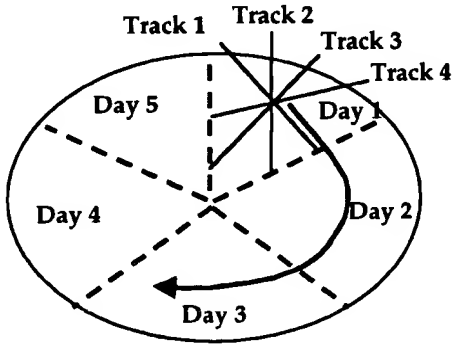

Fig. 1. Tumor injection template used for ONYX-015 administration in this trial. Tumors were injected with 10^{10} pfu per treatment administration following this template as described in the "Materials and Methods" section. Six to eight needle tracks were made radially out from the puncture site; virus was administered equally along the length of the needle tracks. The majority of the viral dose was administered at the tumor periphery and at the interface between normal tissue and tumor. This approach was carried out each day from puncture sites that were equally spaced out and encompassed the entire tumor mass.

The degree of response within injected tumors was categorized as follows: complete regression, complete disappearance of measurable tumor; partial regression, ≥50% but <100% decrease in cross-sectional tumor area; minor response, <50% but ≥25% decrease in tumor area; stable disease, <25% decrease and [mt]25% increase in tumor area; progressive disease, ≥25% increase in tumor area *versus* the baseline area. The time-to-injected tumor progression was defined as the time from treatment initiation to an increase of ≥25% in the nonnecrotic cross-sectional tumor area. To adequately assess the correlation between the effects of ONYX-015 injection within the injected tumor and predictive factors (*e.g.*, p53 status), patients who received less than two cycles of treatment because of either development of comorbid medical conditions (n = 6) or progression at noninjected sites (n = 7) were not evaluable for this analysis. Investigators and radiologists were blinded to the final *p53* gene status and neutralizing antibody titer of the patients at the time of tumor assessment.

Additional Follow-Up after Treatment Initiation. Neutralizing antibody titers were repeated every 4 weeks. Injection site biopsies between days 5 and 22 of the first treatment cycle were optional, based on patient consent because of ethical considerations. These biopsies were analyzed for E1A protein expression (the earliest gene product expressed) and viral replication by *in situ* hybridization. Routine blood testing (complete blood count, electrolytes, blood urea nitrogen, creatinine, and liver function tests) was repeated every 3 weeks.

***p53* Gene Sequencing.** Pretreatment tumor biopsies were taken for p53 sequencing from the recurrent tumor mass that was to be injected. Exons 5–9 were sequenced completely during the first two-thirds of the trial. Mutations were considered functionally significant if present in the Soussi database. Exons 2–11 were assessed by p53 gene chip technology during the final one-third of the trial. Because certain gene deletions can be missed by gene chip analysis (*i.e.*, a wild-type sequence is reported despite a functionally significant mutation), wild-type p53 gene sequences by gene chip analysis required confirmatory sequencing to be validated.

***In Situ* Hybridization for Adenoviral DNA.** *In situ* hybridization for adenoviral DNA was carried out on biopsy samples to determine the extent of replication of ONYX-015 in both tumor and adjacent normal tissues as described previously (14). Briefly, *in situ* hybridization was performed on formalin-fixed, paraffin-embedded tissue, cut into 5-μm sections. Slides were deparaffinized in xylenes, hydrated through ethanols, digested with proteinase K, and postfixed in 4% paraformaldehyde. Hybridization was carried out overnight at 37°C with 0.5 μg/ml biotinylated adenovirus DNA probe (Enzo Diagnostics, Inc., Farmingdale, NY). After three successive washes in 1× SSC at 55°C, an alkaline phosphatase conjugated-antibiotin antibody (Vector Laboratories) was applied. NBT/BCIP was used as the chromagen, and slides were counterstained with nuclear Fast Red.

E1A Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and hydrated. Slides were subjected to antigen retrieval at 120°C for 10 min in citrate buffer and incubated with an adenovirus type-2 E1A antibody (Clone M73; Calbiochem) for 90 min at room temperature. This was followed by incubation with a biotinylated goat antimouse secondary antibody, and streptavidin/horseradish peroxidase conjugate.

Determination of Neutralizing Antibody Titers. Patient and control samples were incubated at 55°C for 30 min to inactivate complement. Clinical plasma samples previously determined to produce high, midrange, and negative titers were designated as plasma controls. Each dilution was mixed with adenovirus stock at a titer prequalified to produce 15–20 plaques per well of a 12-well dish in DMEM growth medium. The patient's samples and controls were inoculated for 1 h at room temperature and applied to 70–80% confluent JH393 cells in 12-well dishes. After 2 h of incubation at 37°C, 5% CO_2 plasma-virus mix was removed and 2 ml of 1.5% Agarose in DMEM were added to each well. Plates were read on day 7 postinoculation by counting the number of plaque-forming units per well. The titer of neutralizing antibody for each sample was reported as the dilution of plasma that reduced the number of plaques to 60% of the number of plaques in the virus control without antibody.

RESULTS

Patient Characteristics. A total of 37 patients were enrolled into one of two protocols to receive either 5 consecutive injections of ONYX-015 over 5 days or 10 consecutive twice-a-day injections of ONYX-015 over 5 days for 2 weeks. To adequately assess the correlation between the effects of ONYX-015 injection within the injected tumor and predictive factors (*e.g.*, p53 status), patients who received <2 cycles of treatment because of either development of comorbid medical conditions (n = 6) or progression at noninjected sites (n = 7) were not evaluable for this analysis. This report focuses on the 24 patients evaluable for response. Baseline patient characteristics were typical for this end-stage patient population (Table 1). Most patients were male (71%). The median age was 58.5 years, and all of the patients had a Karnofsky performance status of ≥70. Patients were heavily pretreated in most cases; 88% of patients had received two or more previous therapeutic modalities, and 54% had received three previous modalities. The most common site of the patients' injected recurrent tumor was the cervical area. Injected tumors had a median diameter of 3.38 cm (range, 1–7 cm) and a median cross-sectional area of 11 cm^2 (range, 1.1–39 cm^2). Only one patient had a distant metastasis present outside of the head and neck region. Patients were relatively immunosuppressed. Delayed-type hypersensitivity skin-testing reactivity to common antigens was below the normal range in 70% of patients, and the median CD4 cell count was 339 (range, 126–1318).

Treatment Characterization. Patients received a median of 2.5 cycles of therapy (range, 2–8 cycles) while remaining on study for a median of 41 days (range, 15–287 days). Nineteen evaluable patients were treated on the five-daily injection regimen, whereas five evaluable patients were treated on the hyperfractionated dosing regimen.

Posttreatment Histology. Tumor biopsies were obtained between day 5 and day 22 of the first cycle of treatment (1–17 days after the final injection of ONYX-015). Each patient (n = 24) evaluable for response (received ≥2 cycles) underwent biopsy at varying time points. Time points of biopsies were 1–3 days (n = 7 patients), 7–10 days (n = 4 patients), and 14–17 days (n = 10 patients) after the last injection. Both tumor tissue and normal tissue were present in all of the evaluable biopsies posttreatment. Viral presence was assessed by

Table 1 *Baseline patient characteristics*

	n (%)
Age (yr)	
Median	58.5
Range	49–74
Gender	
Male	17 (71)
Female	7 (29)
KPS[a]	
90–100	12 (50)
70–80	12 (50)
Prior therapy	
Surgery	18 (75)
Radiotherapy	23 (96)
Chemotherapy	16 (67)
≤2 modalities	21 (88)
≥3 modalities	13 (54)
Location of recurrence	
Cervical (including lymph node)	15 (63)
Oropharynx	3 (13)
Other	6 (24)
Tumor size (max. diameter, cm)	
Mean	3.4 (±2.1)
Median	3.38
Range	1.0–7
Baseline neutralizing antibody levels	
<1:20	10 (42)
≥1:20	14 (58)
CD4 cell counts (cells/μl)	
Median	339
Range	126–1318
p53 gene status	
Mutant	12
Wild-type	7
Indeterminant	5

[a] KPS, Karnofsky performance status; max., maximal.

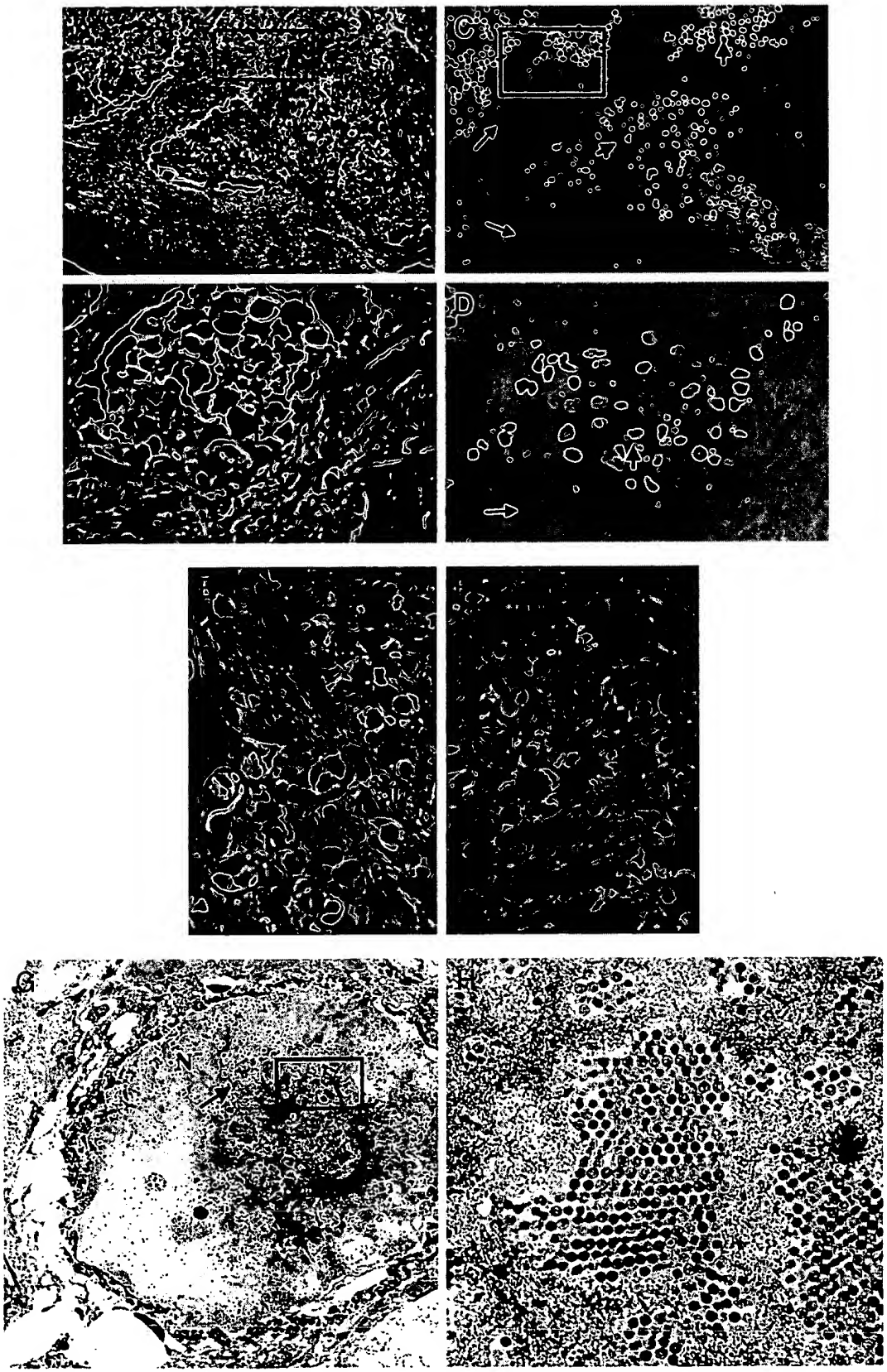

Fig. 2. Evidence of selective intratumoral replication of ONYX-015. *In situ* hybridization for adenoviral DNA on a tumor biopsy specimen obtained on day 8 after treatment initiation with ONYX-015 for 5 consecutive days. The specimen stained with H&E demonstrates viral-induced CPEs (*arrow, A*) and neutrophil infiltration (*arrow, B*) within tumor tissue only. *In situ* hybridization for adenoviral DNA demonstrates replication of ONYX-015 within nests of tumor cells (*short wide arrow(s), C* and *D*) but not within normal tissues (*thin arrow(s), C and D*). Immunohistochemical staining for E1A protein was also demonstrated (*E* and *F*). Electron microscopy represented in previous publications (46) confirms the presence of intranuclear replicating viral particles (*G*) and pseudo-crystalline arrays (*H*) within tumor cells.

Table 2 *Response after ONYX-015 injection related to study variables*

Response was assessed by radiological scanning in 22 patients and by physical exam in 2 patients.

Patient group	Total no.	No. of Patients					P^b
		CR[a]	PR	MR	SD	PD	
p53 gene sequence							
Mutant	12	2	2	3	3	2	0.017
Wild type	7	0	0	0	4	3	
Not evaluable	5	0	1	0	2	2	
Neutralizing antibodies (baseline)							
Positive	14	1	2	2	7	2	0.76
Negative	10	1	1	1	2	5	
Tumor diameter (maximum)							
<3 cm	9	0	2	0	3	4	0.34
≥3 cm	15	2	1	3	6	3	
CD4 cell count (cells/μl)							
<500	14	2	1	1	5	5	1.00
≥500	7	0	1	1	3	2	
Not evaluable	3	0	1	1	1	0	
Treatment regimen							
Standard	19	2	2	2	6	7	0.71
Hyperfractionated	5	0	1	1	3	0	

[a] CR, complete response; PR, partial response; MR, minor response; SD, stable disease; PD, progressive disease.
[b] *P* based on comparison of tumors demonstrating antitumor activity (CR, PR, MR) *versus* no activity (SD, PD) for each evaluable subgroup (Fisher's exact test).

in situ hybridization for adenoviral DNA (specific nuclear staining was required) and by assessment of CPEs on H&E-stained slides (Fig. 2, *A* and *B*).

Biopsies were positive for specific adenoviral DNA staining within the tumor as follows: 5 of 7 on days 1–3 after the last injection; 2 of 4 on days 7–10; and 0 of 10 on days 14–17. The intensity and distribution of staining varied between samples, with up to 25% of tumor cells showing evidence of viral presence in samples staining positive (Fig. 2, *C* and *D*). In contrast, normal skin and mesenchymal tissue within the biopsies were uniformly negative ($n = 21$) by *in situ* hybridization (Fig. 2, *C* and *D*). Comparison of ONYX-015 presence in tumor tissue before day 14 (7 of 11 positive) *versus* normal tissue (0 of 11 positive) was a significant difference ($P = 0.01$). Immunohistochemical staining for adenoviral E1A protein confirmed expression within *in situ* hybridization-positive tumor cells (Fig. 2, *E* and *F*). Viral presence was further confirmed by electron microscopy (Fig. 2, *G* and *H*). Areas of viral presence within the tumors were associated with areas of CPEs and necrosis as seen by neutrophil infiltrate (Fig. 2, *A* and *B*). CPEs were not seen within normal tissues. Thus, the high concentration of ONYX-015 in malignant tissue ≥24 h after the last injection is likely related to selective viral replication.

Abnormalities in p53 were detected in all of the tumors demonstrating viral presence. On days 1–3 after the last injection, four of five p53 mutant biopsies showed viral presence. Two tumors without p53 mutation were biopsied; one was negative for viral presence, whereas the other showed very focal viral presence within an otherwise negative tumor specimen. p53 immunohistochemical staining of this tumor sample documented focal elevated expression within a small nest of tumor cells (<5% of the total), consistent with a p53 abnormality; therefore, focal replication or infection may have occurred within a small focus of cells with abnormal p53. p53 mutations may not be detected by DNA sequencing if present in less than 25% of the cells in the biopsy sample.[6]

Tumor-specific Response. ONYX-015 injection induced a 25–100% response of the injected tumor mass in 8 (33%) of 24 cases (Table 2): two complete (8.3%), three partial (12.5%), and three minor (12.5%) regressions were observed. Normal peritumoral tissue did not appear affected by physical exam in any case, despite direct injection with ONYX-015 (Fig. 3). The intent-to-treat objective regression rate (≥50%) for all of the 37 patients receiving any treatment was 14%.[7]

Of the eight tumor regressions observed, two partial and one minor regression were confirmed approximately 4 weeks later. Confirmation of the other five regressions at 4 weeks after initial response characterization was not possible because of injected-tumor progression ($n = 2$), death from unrelated causes ($n = 1$), patient decision to withdraw from the study ($n = 1$), or distant noninjected tumor progression ($n = 1$) just prior to the 4-week confirmatory evaluation.

Correlation of Tumor Response with *p53* Gene Status. A significant correlation was demonstrated between the induction of tumor response after necrosis and the *p53* gene status of the tumor (Table 2). Seven (58%) of 12 p53 mutant tumors underwent significant necrosis and achieved significant response, whereas none of the 7 p53 wild-type tumors achieved a response ($P = 0.017$). An evaluable *p53* gene sequence could not be obtained from five tumors. Neither the baseline neutralizing antibody status (positive or negative), nor exposure to prior radiotherapy, nor the baseline tumor size (maximal diameter < or ≥3 cm.), nor Karnofsky performance status (≥90 *versus* <90) correlated significantly with response (Table 2).

Time-to-Tumor Progression. Tumor progression was rapid in most cases. The median time to progression at the injected tumor site was 51 days (range, 21–114 days). Six (25%) of 24 patients were without progression of the injected tumor after 3 months on study (Fig. 4). The time to progression of injected p53 mutant tumors (median, 56 days)was delayed compared with p53 wild-type tumors, although not significantly (median, 21 days; Fig. 4; $P = 0.28$).

Humoral Immune Response. Approximately 60% of patients had positive neutralizing antibody titers at baseline. Within 3 weeks of treatment initiation, all of the patients had positive neutralizing antibody titers and significant antibody titer increases (Table 3). There was no correlation between baseline neutralizing antibody titers and induction of tumor response (Table 2).

DISCUSSION

p53 mutation is the most common genetic abnormality identified in human cancer (37), and it is frequently associated with both a de-

[6] Unpublished observations.

[7] J. Nemunaitis, F. Khuri, I. Ganly, J. Arseneau, J. Kuhn, T. McCarty, S. Landers, L. Rommel, C. Heise, B. Randlev, T. Reid, S. Kaye, and D. Kirn. A Phase II trial of intratumoral injection of ONYX-015 in patients with refractory head and neck cancer. J. Clin. Oncol., in press.

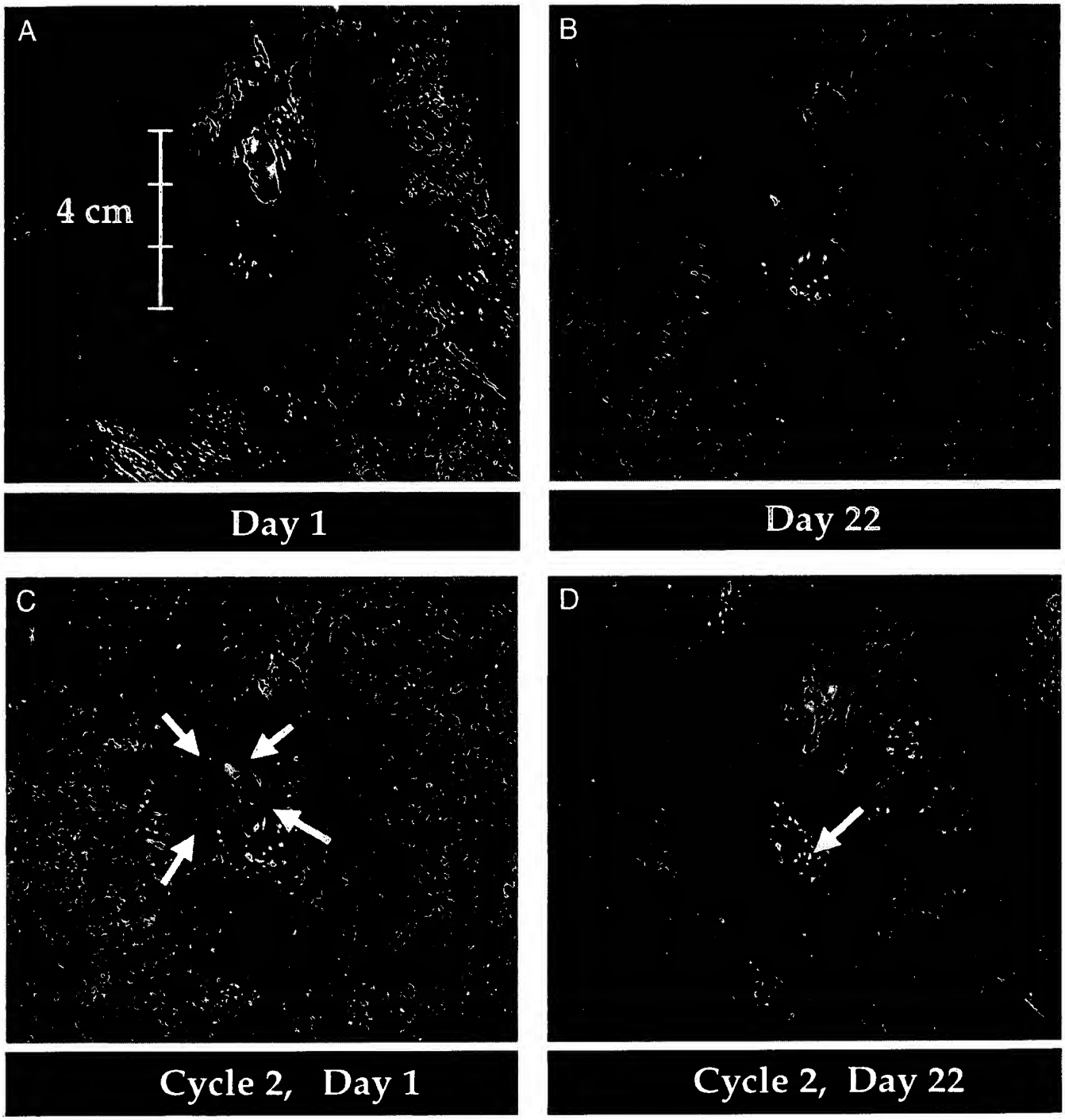

Fig. 3. Evidence of selective tumor destruction after injection of ONYX-015 into both tumor tissue and normal tissue. This patient had a 4-cm left-neck recurrence after prior surgery, radiotherapy, and chemotherapy (*A*). The tumor was partially wrapped around the carotid artery and was, therefore, unresectable. ONYX-015 was injected into the tumor mass and surrounding normal tissues as demonstrated in Fig. 1. On day 22 after treatment initiation, a complete response was evident both clinically (*B*) and radiographically; no damage to the injected peritumoral normal tissue is evident. On days 22–26, a second 5-day course of ONYX-015 was injected entirely into the normal tissue adjacent to the tumor bed [*arrows*, injection locations (*C, Cycle 2, Day 1*; *D, Cycle 2, Day 22*)]. Despite direct injection of the entire dose of ONYX-015 into the normal tissue for 5 consecutive days, the normal tissue was clinically unaffected on day 44 (*D*). *Arrow*, granulation tissue healing into the ulcerated area.

creased responsiveness to standard chemotherapeutic agents and a poor prognosis (6, 38, 39). Novel therapies that can target these resistant tumors are needed. We report that ONYX-015 can cause CPEs in recurrent and refractory p53 mutant squamous cell tumors of the head and neck. ONYX-015 presence was not detected in peritumoral normal tissue, despite direct injection. In addition, ONYX-015 induced p53 mutant tumor-specific response in association with necrosis. CPEs were not documented in normal tissue nor in tumors containing wild-type p53 genotype. ONYX-015 seems to be the first therapeutic agent specifically designed to target p53-deficient tumor cells that has demonstrated selectivity in patients. Future clinical trials will demonstrate whether this selectivity for p53 mutant tumor generalizes to other cancer patient populations. ONYX-015 is also the first genetically engineered replication-competent virus to demonstrate selective intratumoral activity in patients.

The role of the immune response to replicating viral agents is

unclear. This will best be answered in clinical trials because of the lack of an immunocompetent animal model that supports efficient adenoviral replication (40, 41). Neutralizing antibody titers either before or after treatment were not predictive for antitumor activity. Although encouraging, this finding does not rule out an inhibitory role for neutralizing antibodies during a longer-term treatment or after other routes of administration (*e.g.*, i.v.). Future studies will be needed to better define the role of antibodies and whether their suppression would be beneficial. The role of cell-mediated immunity in either increasing or decreasing the antitumor activity in these patients is still unclear. These end-stage head and neck cancer patients were relatively immunosuppressed. CD4 cell counts were less than 500 (per μl) in 65% of the patients and less than 200 in 25%. Delayed-type hypersensitivity skin reactivity to common antigens was low in 70% of patients. Therefore, cell-mediated immunity may play less of a role than in more immunocompetent patient populations. The time course and magnitude of immune cell infiltration into tumors after injection will best be determined by histological assessment of the entire tumor mass (*e.g.*, after surgical resection) at varied time points after treatment. On the basis of those results, immunomodulatory strategies might be developed. Finally, antiviral cytokines may also affect adenoviral replication and/or spread (42, 43).

Despite the encouraging biological activity demonstrated with ONYX-015 in this clinical trial, clinical benefit was not seen in the majority of patients. Tumor progression was rapid in the vast majority of patients, even for tumors that underwent substantial necrosis after treatment. These patients were heavily pretreated and were end-stage in most cases; the life-expectancy is 3–4 months in this patient population (44, 45). Additionally, because patients who progressed within two cycles at noninjected sites were excluded, these results cannot entirely rule out the possibility of a more beneficial response in patients with multiple slower-growing tumors. The true clinical benefit of intratumoral injection with ONYX-015 as a monotherapy will, therefore, need to be determined in randomized trials and, possibly, in earlier stage patients.

Future approaches also include the addition of therapeutic genes with antitumoral effects to ONYX-015 (*i.e.*, using ONYX-015 as a delivery vehicle), so-called "armed therapeutic viruses." ONYX-015 has several favorable characteristics as a vector for gene delivery: inherent antitumoral activity and selectivity; potential amplification of the transgene leading to high level expression; and enhanced intratumoral spread *versus* nonreplicating vectors. If these approaches are successful, viral therapy with genetically engineered viruses may become a novel therapeutic platform for the treatment of cancer.

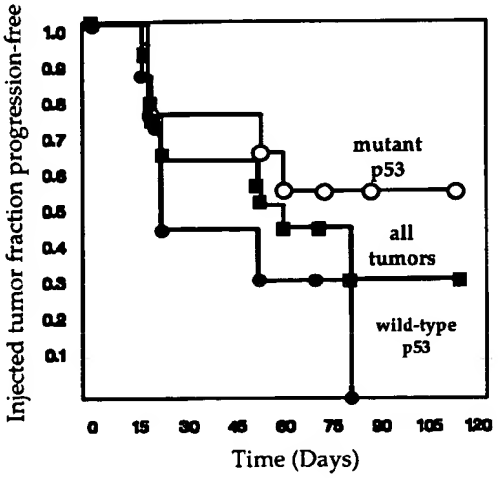

Fig. 4. Kaplan-Meier analysis of the time-to-progression of p53 mutant *versus* p53 wild-type tumors injected with ONYX-015.

Table 3 *Humoral immune response to intratumoral injection of ONYX-015*

Time point	Positive neutralizing antibody titers (%)	Neutralizing antibody titer (median, range)
Baseline	60	112 (0–8,847)
End of cycle 1 (day 22)	100	10,908 (175–81,920)
End of cycle 2 (day 44)	100	12,363 (225–71,425)

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